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Genetic Diversity in Clinical Isolates of the Dimorphic Fungus *Blastomyces dermatitidis* Detected by a PCR-Based Random Amplified Polymorphic DNA Assay

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***Blastomyces dermatitidis* is a dimorphic fungus causing localized or systemic infection in areas where the organism is endemic in the central and southeastern United States. In this study, 19 independent isolates of *B. dermatitidis* from Little Rock, Ark., were grouped into three classes based on restriction fragment length polymorphism patterns in mitochondrial DNA with a heterologous probe from *Histoplasma capsulatum*. One large class of 15 isolates and two smaller classes (classes 2 and 3), each consisting of two isolates, were observed in *Bgl*II digests. Strain-specific arrays of PCR-amplified DNA products were obtained with arbitrarily selected primers (18 to 29 nucleotides long; G+C contents, 33 to 56%). In the large class 1 group, 13 isolates could be differentiated by the random amplified polymorphic DNA (RAPD) method with various primers. The two remaining class 1 isolates were obtained from the same patient and produced identical RAPD arrays. Dissimilar RAPD patterns were obtained from the smaller class 2 group but not from the class 3 isolates. Significant genetic diversity in clinical isolates of *B. dermatitidis* was observed; this may underscore a similar environmental diversification. Further application of the typing techniques may provide significant insight into the epidemiology of blastomycosis and aid in the assessment of specific virulence phenotypes.**

Blastomycosis is one of the principal endemic systemic mycoses occurring primarily in the south central and upper midwestern United States (4). The disease is caused by the thermal dimorphic fungus *Blastomyces dermatitidis*, which dwells as a saprophyte in the environment or at 25°C but converts to the parasitic yeast phase in vivo following inhalation of microconidia or in vitro at 37°C. Most cases are sporadic, although several outbreaks have been described (4, 10, 11). The infectious agent has proved difficult to isolate from soil and has been successfully cultured in rare circumstances, generally during outbreaks (11, 24). This is the only evidence which links the disease to particular environmental reservoirs.

In immunocompetent individuals, infection causes a mild influenza-like or atypical pneumonia syndrome (4, 20) but can also result in long-term carriage of the organism with the potential for reactivation following the initial exposure (6). Blastomycosis is also associated with immune dysfunction, including infection in transplant recipients (5), individuals receiving maintenance immunosuppressive therapy (5, 17), and patients with AIDS (17). As the population of immunocompromised patients continues to rise, endogenous reactivation of latent infection, as demonstrated in several patients with AIDS who developed blastomycosis in regions outside of the area where *B. dermatitidis* is endemic (6, 17), may become a life-threatening complication.

For epidemiologic studies, it is important to distinguish among individual strains of *B. dermatitidis* which will provide enhanced sensitivity for more detailed analysis of infection mechanisms or pathogenesis. In recent studies, restriction fragment length polymorphisms (RFLPs) in ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) has emerged as a method for typing of *B. dermatitidis* isolates, as well as other fungal pathogens, including *Histoplasma capsulatum* (23, 25) and *Candida albicans* (12, 18, 21). Fraser and coworkers applied RFLP typing by Southern blot analysis to evaluate *B. dermatitidis* isolates from two patients (7).

More recently, a new PCR-based method of DNA fingerprinting, known as the random amplified polymorphic DNA (RAPD) assay (26, 27), has emerged as a sensitive technique for microbial strain identification and classification (1, 2, 9, 14, 26, 27). Strain-specific arrays of DNA fragments or fingerprints are generated from arbitrary oligonucleotides which nearly or completely match genomic sequences and are thus used to prime DNA synthesis during PCR amplification. In this report, we describe the use of RFLP typing of 19 isolates of *B. dermatitidis* with mtDNA probes and compare this classification to strain typing with the RAPD PCR method to generate DNA fingerprints from genomic sites within the fungal DNA that fortuitously match the arbitrary primer.

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MATERIALS AND METHODS

Organisms and culture conditions. Mycelial-phase organisms were cultured in 2% glucose–1% yeast extract with 8.4 μg of cysteine per 1,000 ml (GYE). Clinical isolates 1 through 19 were obtained from Robert Bradsher, University of Arkansas for Medical Sciences, Little Rock, and were identified as *B. dermatitidis* by histopathologic and culture examinations.

Preparation of genomic DNA. Whole-cell DNA was extracted from a 600-ml volume of cultured mycelial-phase organisms which was filtered and then broken by grinding under liquid nitrogen with a prechilled mortar and pestle. The mycelial powder was transferred to lysis buffer, consisting of 0.05 M Tris-HCl (pH 8.0), 0.063 M EDTA, and 2% sodium dodecyl sulfate (SDS), and stirred for 1 h. Cell debris was pelleted, and the supernatant was made 2 M in potassium acetate by addition of an 8 M stock and incubated on ice for 30 min. Centrifugation removed a large gelatinous protein-carbohydrate mass, and the recovered

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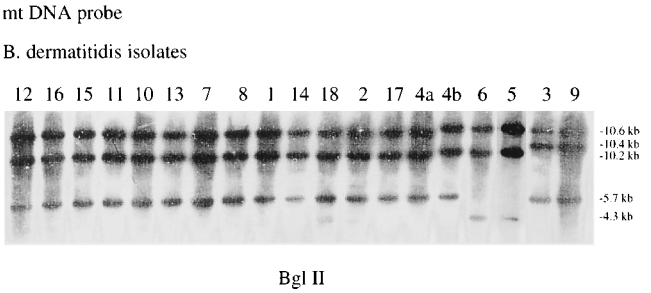

FIG. 1. RFLPs at the mtDNA locus in *B. dermatitidis* isolates. Genomic DNA was digested with *Bgl*II and evaluated for RFLP at the level of mtDNA by using a set of three heterologous probes representing the entire *H. capsulatum* mitochondrial genome (23).

supernatant was precipitated overnight at −20°C by addition of 8 M ammonium acetate to 2 M and an equal volume of isopropanol. Nucleic acids were recovered by centrifugation, resuspended in 10 ml of 20 mM Tris-HCl–1 mM EDTA–20 mM NaCl (pH 8.0), and digested with RNase A (20 μg/ml) for 30 min at 37°C, and the incubated with proteinase K (50 μg/ml) for an additional 30 min. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1 ratio) and precipitated in ethanol. DNA was further purified by centrifugation in CsCl-ethidium bromide at 51,000 rpm (Beckman L-70 UC) and 17°C overnight. The DNA was dialyzed against TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and resuspended to approximately 1 μg/μl. Nucleic acid concentrations were determined with a Shimadzu UV160U spectrophotometer. The DNA from this protocol was utilized for Southern blot analysis of RFLPs at the rDNA and mtDNA loci and in some of the RAPD PCR typing.

For routine RAPD PCR typing, nucleic acids were prepared by the boiling-lysis method of Woods et al. (28) with 5-ml stationary-phase cultures of mycelial-phase organism grown in GYE. Nucleic acids were resuspended in 60 μl of distilled water, and concentrations were estimated by electrophoresis of 5-μl samples on 0.7% agarose gels stained with ethidium bromide. Typically, 1 to 5 μl of material was utilized in each PCR.

Analysis of RFLPs. Genomic DNA (2 to 5 μg) was digested at 37°C for 1 h or overnight with restriction endonucleases *Eco*RI, *Bgl*II, *Hin*dIII, and *Pst*II. DNA segments were separated by electrophoresis on an 0.8% agarose gel in TBE (0.89 M Tris, 0.089 M boric acid, 2 mM EDTA, pH 8.0) containing 1 μg of ethidium bromide, photographed, and transferred by the method of Southern (22). Sizes of all DNA fragments were estimated by coelectrophoreses of *Hin*dIII fragments of lambda DNA. Blots were hybridized with a pooled probe representing mtDNA from *H. capsulatum* (23) or with an 8.3-kb *Not*I fragment containing an entire rDNA repeat cloned from *H. capsulatum* (23) labelled by nick translation (19). Hybridizations were performed at 65°C in an aqueous hybridization mixture containing 7% SDS, 1% bovine serum albumin, 1 mM EDTA, 0.14 M monobasic sodium phosphate, 0.36 M dibasic sodium phosphate, and 10^6 cpm of nick-translated probe per ml. Blots were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.1% SDS. Two additional washes were performed at 65°C in 0.2× SSC–0.1% SDS.

Conditions of the RAPD-PCR assay. PCR was performed with 20-μl volumes in a Precision Thermal Cycler (GTC-2) programmed as follows: 4 cycles at 94°C for 5 min, 40°C for 5 min, and 72°C for 4 min in a low-stringency amplification followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min in a high-stringency amplification and then incubation at 72°C for 10 min. All reaction conditions were optimized with a randomly chosen isolate, no. 1.

Optimal DNA concentrations in the assays were 2.4 ng/μl in reactions performed in 20 μl. The M13/pUC reverse, Sp6 promoter, and Histo primers were all used at 10 nM. The RAPD assay was performed with 0.1 U of thermostable *Taq* polymerase (Perkin Elmer Cetus) and dCTP, dGTP, dATP, and dTTP, each at 0.250 mM, in 10 mM Tris-HCl–50 mM KCl–6.5 mM $MgCl_2$–0.001 U of gelatin per volume.

In experiments in which ^{32}P-labelled, PCR-amplified fragments were analyzed, 0.5 to 1 μCi of [α-^{32}P]dATP was included in the 20-μl PCR mixture. Products were electrophoresed on 8% urea–polyacrylamide gels for 5 h at 300 V, mounted, and exposed to XAR5 X-ray film at −80°C for 6 h or overnight prior to development.

RESULTS

Classification of *B. dermatitidis* isolates by RFLPs at the mtDNA locus. Nineteen independent isolates of *B. dermatitidis* obtained from patients in Little Rock, Ark., were evaluated for RFLPs at the mtDNA locus (Fig. 1). All of the isolates possessed the same 10.6-kb *Bgl*II fragment detectable with a group

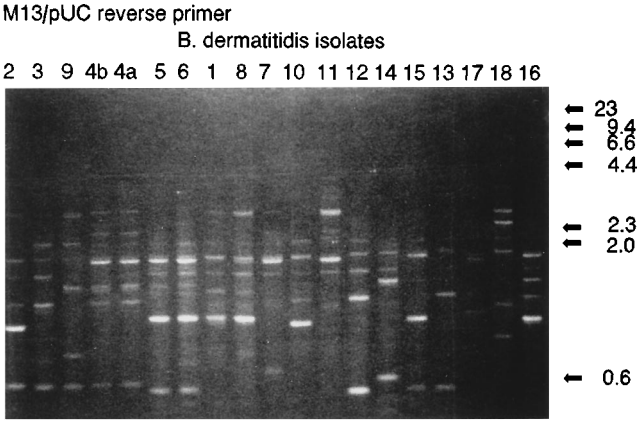

FIG. 2. Analysis of *B. dermatitidis* isolates in the RAPD PCR assay with the M13/pUC reverse primer by gel electrophoresis and staining. DNA from *B. dermatitidis* isolates was amplified in 20-μl reaction volumes containing 2.4 ng of genomic DNA per ml, 5 mM $MgCl_2$, and 10 nM M13/pUC reverse primer as described in Materials and Methods. The gel was stained with ethidium bromide. The numbers on the right are molecular sizes in kilobases.

of three heterologous probes representing the entire *H. capsulatum* mitochondrial genome (23). Moreover, all of the isolates had a 10.2-kb *Bgl*II fragment by this analysis, except isolates 3 and 9, which had a 10.4-kb band. Additionally, all of the isolates contained a 5.7-kb *Bgl*II band, except isolates 5 and 6, which had a 4.3-kb fragment. These findings suggested that the isolates can be organized into three classes on the basis of mtDNA polymorphism: class 1, consisting of 15 isolates; class 2, consisting of isolates 3 and 9; and class 3, containing isolates 5 and 6. This classification is also supported by analysis of RFLPs at the rDNA locus with a heterologous probe representing an entired rDNA repeat from *H. capsulatum* (23) and by additional RFLP patterns obtained with *Eco*RI, *Pst*II, and *Hin*dIII (data not shown).

Analysis of *B. dermatitidis* isolates by the RAPD PCR assay with the M13/pUC reverse primer. RAPD PCR analysis with the M13/pUC reverse primer, a 23-bp primer with a G+C content of 43% (see Table 1), produced an array of amplified fragments primed from *B. dermatitidis* genomic DNA ranging from 0.6 to 3.0 kb (Fig. 2), demonstrating significant genetic diversity among the class 1 isolates (all isolates except 3, 9, 5, and 6). Within class 1, unique DNA fingerprints were generated in this size range, with the exception of isolates 1 and 8 and paired isolates 4a and 4b, which were cultured from the same individual at different times and which supported identical arrays of amplified fragments. As seen in Fig. 2, the class 2 isolates, no. 3 and 9, differed in extension fragments in the 0.8- to 1.8-kb range and were clearly distinct, while the class 3 representatives, no. 5 and 6, generated identical amplification profiles with this primer. These findings were further supported at single-nucleotide resolution by labelling of amplified products during PCR, followed by electrophoresis on sequencing gels and autoradiography (see below).

Amplification of useful long (≥25-nt) and short (<25-nt) primers for evaluation of the *B. dermatitidis* genome. To optimize the RAPD PCR method for *B. dermatitidis*, isolate DNAs were tested with nine different primers for amplification that resulted in arrays of 3 to 15 prominent bands in the range of 0.3 to 4.0 kb (Table 1). Longer primers (≥25 nucleotides [nt]) that provided good amplification profiles within this size range included three primers that have been used to study higher plants (26) and *H. capsulatum* (8) and were designated in this

TABLE 1. Arbitrary primers tested for PCR amplification

Primer size (bp), amplification quality, and designation	Sequence	% G+C
≥25		
Good		
Primer 2	GGTTATCGAAATCAGCCACAGCGCC	56
Histo 1	GGCCATAGAGTCTTGCAGACAAACTGC	52
Histo 2	AACGTTCATGATAACTTCTGCTCTTCATC	38
Histo 3	AAGCTTGCATTTGTGTTCCTTGATAAGTG	38
Little (if any), primer 1	GATGAGTTCGTGTCCGTACAACTGG	52
≤25		
Good		
M13/pUC reverse	AGCGGATAACAATTTCACACAGG	43
Sp6 promoter	ATTTAGGTGACACTATAG	33
Little (if any)		
M13/pUC forward	CCCAGTCACGACGTTGTAAAACG	52
T7 promoter	TAATACGACTCACTATAGGG	40

analysis the Histo 1, 2, and 3 primers. The G+C contents of these long primers ranged from 38 to 56%, although one primer having a similar size and nucleotide content (primer 1) proved unsatisfactory. Short (<25-nt) primers were also evaluated for the ability to discriminate among *B. dermatitidis* isolates. Good amplification was achieved with two short primers, the M13/pUC reverse and Sp6 promoter primers (33 to 43% G+C content). Thus, six of the nine primers tested provided PCR-generated arrays that were useful for analysis of genetic diversity within clinical isolates or natural populations.

To assess the reliability of the RAPD PCR technique for discrimination among isolates of *B. dermatitidis*, three representative members of class 1 were analyzed with several different primers (Fig. 3). As seen in Fig. 3, the short (<25-nt) Sp6 promoter and M13/pUC reverse primers and longer (≥25-nt) primer 2 were used to amplify different genetic regions

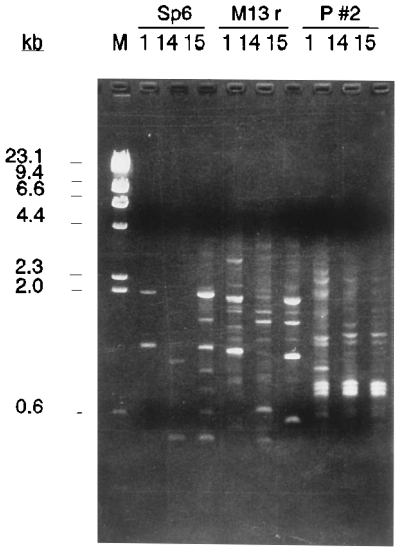

FIG. 3. Multiple-primer analysis of three representative class 1 isolates of *B. dermatitidis*. Genomic DNAs from three representatives of class 1 (no. 1, 14, and 15) were amplified under the conditions described in Materials and Methods with several primers (Sp6 promoter, M13/pUC reverse, and primer 2). A marker lane (M) was electrophoresed in parallel, and the sizes of *Hin*dIII-restricted lambda phage DNA fragments are indicated. The products range in size from 0.4 to approximately 4.2 kb.

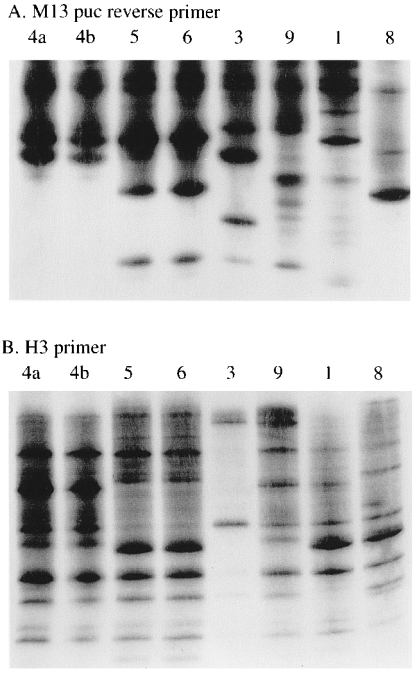

FIG. 4. RAPD PCR analysis of representatives of all RFLP classes with the M13/pUC reverse primer (A) and the Histo 3 primer (B). Genomic DNAs from class 2 (no. 3 and 9), class 3 (no. 5 and 6), and several representatives of class 1 (no. 1, 4a, and 4b) were amplified with the M13/pUC reverse primer (A) and the Histo 3 primer (B). RFLP class 1 isolates 4a and 4b were isolated from the same patient but cultured at different times during infection and produce identical amplified DNA patterns, suggesting that these isolates are isogenic. Products were electrophoresed on 8% urea–polyacrylamide gels in parallel with *Hin*dIII-digested lambda DNA (size range, 120 bp to 23.1 kb) and *Hin*fII-*Eco*RI-digested pBR322 markers which were labelled by end filling with the Klenow fragment (size range, 75 to 998 bp).

from isolates 1, 14, and 15 to generate distinctive arrays of PCR fragments. The results suggest that all three of the isolates evaluated as shown in Fig. 3 are genetically distinct. Replicates of this experiment were performed with DNA isolated by the boiling-lysis method (28) described for *H. capsulatum*. Similar arrays of amplified products were obtained when the original and replicates were compared (data not shown).

Analysis of all *B. dermatitidis* RFLP classes with the M13/pUC reverse primer and the Histo 3 primer. DNAs from isolates 1, 8, 4a, 4b, 3, 9, 5, and 6 were used as templates for RAPD PCR analysis with the M13/pUC reverse primer (Fig. 4A) to generate ^{32}P-labelled extension fragments which were electrophoresed on denaturing 8% urea–polyacrylamide gels (15). In this analysis, class 2 members 3 and 9 were clearly distinct from themselves and from class 1 isolate 1. However, class 3 isolates 5 and 6 produced identical extension fragment patterns, suggesting a high degree of similarity between these strains at the seven distinct loci primed with the short oligonucleotide. In contrast, RFLP class 1 isolates 1 and 8 were distinct from one another, while 4a and 4b were isolated from the same patient but cultured at different times during infection and produced identical arrays. Additional experiments in which denatured DNA fingerprints were separated on native acrylamide gels to combine single-stranded conformation polymorphisms with PCR-based fingerprints profiles (13) confirmed these findings (data not shown).

These findings were extended and supported by applying the Histo 3 oligonucleotide primer (≥25 nt) in the RAPD PCR

assay. As seen in Fig. 4B, this primer generated fingerprints consisting of approximately 13 to 15 bands ranging in size from 0.3 to 2.1 kb and has a greater potential for discrimination among strains. With this primer, isolates 3 and 9 (class 2) were again different while the remaining strains, 5 and 6 (class 3), provided identical arrays when they were compared. Similar RAPD PCR profiles were observed for isolates 4a and 4b, which produced extension fragments different from those obtained from other class 1 members, i.e., strains 1 and 8.

DISCUSSION

Clinical isolates of *B. dermatitidis* have, in general, been subdivided into a few distinct strains by employing Southern blot-based RFLP analysis of rDNA (6). In the study of Fraser and coworkers, only four isolates from a restricted area were evaluated for strain-specific RFLP differences (7). The results presented in this study represent the largest survey ever of RFLP typing of *B. dermatitidis* isolates, all of which were collected from patients in a limited region of Arkansas. Three classes were observed among 19 clinical isolates as assessed by polymorphisms detected with heterologous *H. capsulatum* mtDNA and rDNA probes.

To explore the potential for variation of *B. dermatitidis* within clinical samplings or, by inference, natural populations, a sensitive and rapid PCR-based assay using arbitrary primers was developed. This study reinforces the advantages of the RAPD PCR technique for examining genome diversity and isolate identification for *B. dermatitidis* previously established for pathogenic, dimorphic fungi such as *H. capsulatum* (9). The technique is sensitive and reproducible and operates with minimal quantities of DNA prepared by a boiling-lysis–phenol extraction method. A distinct advantage of the method resides in its ability to sample diversity throughout the genome rather than at restricted single loci.

At least nine different primers were examined for the ability to generate strain-specific extension products in the RAPD PCR assay, with six primers, ranging in G+C content from 33 to 56%, providing acceptable arrays. In contrast to these findings, short primers with 60 to 70% G+C have worked successfully for *H. capsulatum* (9) and *Helicobacter pylori* (1, 2). These findings are of particular interest for analysis of the *Histoplasma* and *Blastomyces* genomes, for which the G+C contents of nuclear DNAs have been reported to be 47.3 and 48.2%, respectively (3).

Multiple factors are likely to contribute to the high variability in the genome of *B. dermatitidis* observed in the clinical specimens examined in this study and even in local environments where the organism is endemic. For example, the diversity observed in these clinical isolates suggests that *B. dermatitidis* is resident in many soil types or microclimates in Arkansas, an area with the highest incidence of disease in the geographic United States. Genetic drift, exchange between genomes following sexual mating (16), and unique selection pressures impacting on the organism in various environmental niches may influence the genetic structure of *B. dermatitidis* in populations. With the RAPD PCR fingerprinting approach, it is feasible to determine if a predominant strain with a distinctive genotype is responsible for localized outbreaks of blastomycosis (10, 11, 24) and if the distribution of genotypes available from a clinical setting compares with that of the occasional environmental isolates obtained from the same area. Future studies using this sensitive and rapid approach may permit identification of specific virulence-associated genotypes of *B. dermatitidis* which establish infection, induce latency, and re-emerge from latency during reactivation.

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