Soil isolation and molecular identification of *Coccidioides immitis*

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Abstract: The fungal pathogen *Coccidioides immitis*, causative agent of coccidioidomycosis or Valley Fever, was first isolated from the environment in 1932. It has been isolated on numerous occasions since then, but always with the use of a mammalian host. The morphology of *C. immitis* is indistinct from related species, thus, its identification hinges upon its ability to infect and produce spherules in a susceptible animal. In this study, four genetically distinct isolates of *C. immitis* were isolated from soil samples from the San Joaquin Valley without the use of a host. None of these sites had been definitively associated with human infection. The isolates were identified from over 2400 soil isolates from 720 soil samples using *C. immitis* specific primers based on the ITS sequence of ribosomal DNA. They were further typed using molecular markers available for clinical isolates of *C. immitis*.

Key Words: fungi, ITS, microsatellites, molecular typing, Onygenales, ribosomal DNA

INTRODUCTION

The fungus *Coccidioides immitis* (Onygenales, Plectomycetes, Ascomycota) is a human, mammal and reptilian pathogen native to the semiarid soil of the Lower Sonoran Life Zone (Maddy et al 1957). For the most part, hot, dry summers and mild winters characterize these areas. In the United States the endemic region includes the San Joaquin Valley in California, inland San Diego County, Arizona, and parts of New

Mexico, Utah and Texas. *Coccidioides immitis* is also found in Mexico, Central America and parts of South America. *Coccidioides immitis* is a soil saprobe and has been shown to have a selective advantage over other fungal competitors in saline and alkaline soils (Egeberg et al 1964, Elconin et al 1964). The fungus is thought to be associated with creosote bushes, rodent burrows and Amerindian midden soils (Lacy and Swatek 1974). Even when soil sampling has been undertaken in areas thought to be ideal for the growth of *C. immitis*, retrieving the fungus has been unpredictable. Indeed, Elconin et al (1964) noted that over a period of 8 yr in a 40 470 square m parcel of land endemic for *Coccidioides*, the percentage recovery from soil samples ranged from 0% to 43%.

Isolation of the fungus from the soil has been accomplished using various methods (Georg et al 1951, Egeberg and Ely 1956, Omieczynski and Swatek 1967). One of the favored methods for culture purification and identification includes growth in a mammalian host, normally a rodent such as a mouse or guinea pig. This method has the advantage of allowing definitive identification of the fungus and selective culture conditions since only *C. immitis* is known to produce spherules in an infected animal. The isolation method developed by Omieczynski and Swatek (1967) utilized a selective culture medium for isolation, with definitive identification in a mammalian host. According to their work, this isolation method was as successful as a mouse isolation method, however, the mouse isolation method allows for definitive identification at the same time.

The problems inherent in the use of a mammalian host include expense and time; such a method also selects for virulence. There has been a wide range of virulence reported in clinical strains (Friedman et al 1956), leaving open the possibility that there may be an even wider range of virulence in environmental samples. These differences in virulence, in addition to differences in host immune status and genetic predisposition, may play a role in the wide variation seen in disease severity (Kirkland and Fierer 1996). Using DNA variation rather than a mammalian host to identify the organism allows for the possibility of retrieving mildly virulent or avirulent strains.

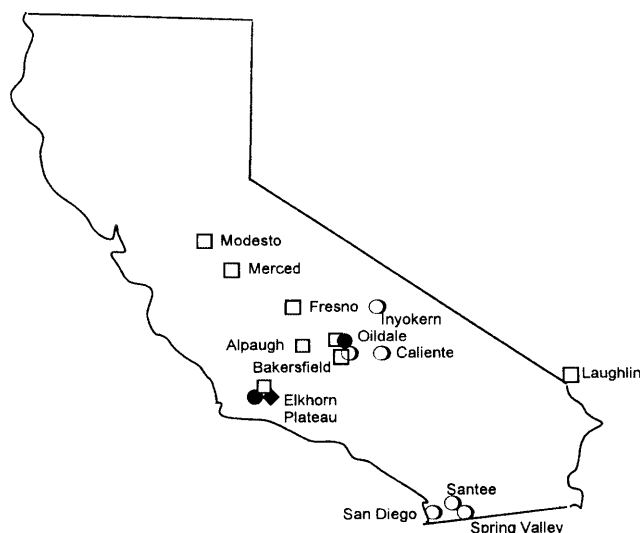Morphologically, *C. immitis* is similar to members of the Onygenales in that it produces rhexolytic ar-

FIG. 1. Map of soil sampling sites during 1994 (squares), 1995 (diamond), and 1997 (circles). Closed shapes indicate where *C. immitis* was recovered.

throconidia which break off from the hyphae by means of a fracture of the intercalary cell (Currah 1985). Its classification in the Ascomycota was problematic since classification of the Onygenales was based largely on characteristics of the sexual states, and a sexual state has never been observed in *Coccidioides*. Given this lack of a sexual state, its taxonomy was not firmly established until sequence of the 18S subunit of ribosomal DNA classified it among the Onygenales (Bowman and Taylor 1993). Additional sequence and biochemical data have confirmed this relationship (Pan et al 1994, Bowman et al 1996).

MATERIALS AND METHODS

Soil isolation and culture.—In spring 1997, two collecting trips were made to areas in California endemic for coccidioidomycosis. They included areas around Bakersfield and Inyokern in Kern County, eastern San Diego County and the Elkhorn Plateau in San Luis Obispo County (FIG. 1). In addition to the 535 soil samples obtained on these two collecting trips, also available for analysis were 45 soil samples obtained in 1994 and the 140 soil samples obtained in 1995. These samples were from throughout the entire San Joaquin Valley and sites near Laughlin, on the Nevada–Arizona border (FIG. 1). Soil locations chosen included sites with animal burrows, sites with evidence of prior indigenous habitation, sites with creosote bushes and sites where human or animal infection had been suspected to occur but had never been proven. Samples were obtained approximately 4–6 wk after the last rainfall in spring 1997. Two soil corers were used of 2.2 cm and 4.4 cm diam. Sample cores included surface soil and core depths between 7.5 and 15 cm (Egeberg and Ely 1956, Kemp 1974). Soil samples were deposited in sterile whirlpak bags and kept at room temperature for storage (Kemp 1974). Also available

for screening were 45 soil samples obtained in fall 1994 and 140 soil samples obtained in fall 1995 that had been kept in sealed whirlpak bags at room temperature during the interval before screening (3 and 4 yr, respectively). They had initially been screened for *C. immitis* after growth under the same growth and culture conditions outlined below, but were not screened using species-specific primers. These cultures were examined microscopically and no *C. immitis* was identified at that time.

The technique for fungal isolation was the double pour, antibiotic (cyclohexamide, streptomycin, chloramphenicol) fortified yeast extract agar method (Omieczynski and Swatek 1967) long utilized for soil isolation of *C. immitis*. Under biosafety level three containment, cultures were grown at 30 C for one wk and then transferred to an ambient temperature of 25 C. All plates were checked weekly for fungal growth. All colonies that were black were discarded. This color has never been noted in a *C. immitis* isolate. If a plate contained morphologically identical isolates and these isolates did not resemble known *C. immitis* morphology, only one of the colonies was tested. When colonies were at least 1 cm in size, one half of the colony was subcultured onto an agar slant containing the initial culture media and one half transferred to 500 μL respiratory lysis buffer (0.1 N NaOH, 1% triton, 1 mM EDTA). Samples in lysis buffer were heated to 90 C for 10 min, conditions that have been shown to kill *Coccidioides* (Burt 1994).

DNA techniques.—DNA extraction was accomplished by manually grinding the fungal sample in lysis buffer plus 27 μL of molecular grinding resin (Genotech, St. Louis, Missouri). This was followed by incubation in a waterbath for 1 h at 65 C. Ground, heated samples were centrifuged at 13 000 rpm for 2 min, and approx 450 μL of supernatant was transferred to a 1.5-mL Eppendorf tube containing 100 μL of Tris (pH 7.5). The tubes were mixed and subsequently maintained at -20 C.

Coccidioides immitis species-specific primers were designed based on the ITS sequences of *C. immitis*, its two closest known relatives, *Uncinocarpus reesii* and *Auxarthron zuffianum* (Pan et al 1994, Bowman et al 1996), and *Gymnoascus longitrichus*, all members of Onygenales and common to the endemic area of *Coccidioides*. The ITS region was chosen due to its high copy number and nucleotide variability. The primer pair, designated ITS C1A (CAT CAT AGC AAA AAT CAA AC) and ITS C2 (AGG CCC GTC CAC ACA AG), was tested against the above Onygenales, several species of *Penicillium* and *Aspergillus,* and 30 clinical isolates of *C. immitis* from California, Texas and Arizona. The primer pair did not amplify any of the non-*Coccidioides* species tested. The primers correctly amplified the 30 clinical isolates of *C. immitis,* at annealing temperatures ranging from 50–62 C (data not shown).

Because there are many reasons that PCR amplification using ITS specific markers might fail, a positive control was needed. We chose fungal specific primers of the 18S subunit of rDNA, RDS 478 (GTG GTT CTA TTT TGT TGG TTT CTA) and RDS 482 (TAG CGC GCG TGC GGC CCA GA), a region that would amplify for all fungi. This region and the *C. immitis* specific ITS region were amplified to-
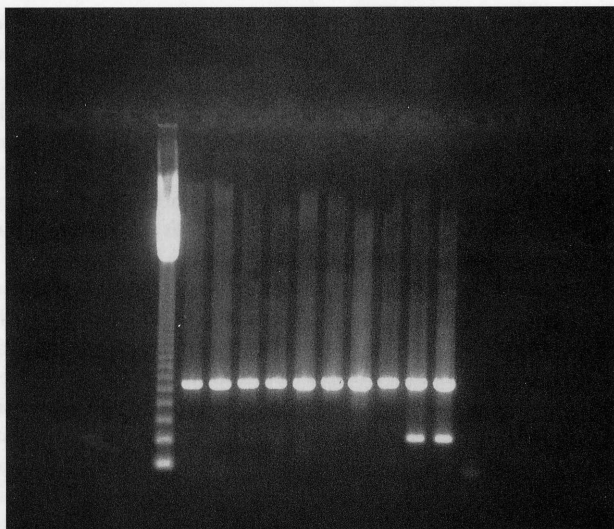
FIG. 2. Agarose gel of various fungi after PCR multiplex with fungal specific and *C. immitis* specific primers. The first lane contains a 123 bp ladder. Lanes with single bands are, in order, *A. zuffianum, U. reesii, G. longitrichus, A. fumigatus, P. jejunum, C. albicans, C tropicalis* and *H. capsulatum*. The following two lanes with two bands each are *C. immitis*—the first, from *C. immitis* culture RMSCC 2484, and the second, *C. immitis* from soil sample HS 3.14.

gether in a multiplex PCR which was determined to yield optimal results at an annealing temperature of 53 C (94 C, 2 min followed by 35 cycles of 94C, 1 min—53 C, 1 min—72 C, 1 min with a final extension of 72 C for 7 min). Thus, for each PCR reaction, one partial 18S band at approximately 600 bp would indicate successful fungal DNA extraction and PCR amplification, and a second band at 223 bp indicated if the fungal DNA was *C. immitis* (see FIG. 2). All samples producing 2 bands of the correct size were sequenced over the ITS region to confirm that they were indeed *C. immitis*. Occasionally a second band of an incorrect size was amplified; in these cases, the 18S rDNA control band (RDS 478/482) was sequenced and thus could be used in conjunction with a BLAST search to ascertain the identity of fungus—generally to a genus level, occasionally to species level.

Microsatellite typing. Individual isolates of *C. immitis* were typed using microsatellite-containing loci developed for population genetic studies (Fisher et al 1999). Using fluorescently labeled dyes for the forward primer of each of 8 primer pairs, the nucleotide length of each PCR product was determined using a TAMRA-labeled size standard and ABI 377 Automated Sequencer (Applied Biosystems).

RESULTS

Of the 535 soil samples obtained in spring 1997, three yielded *C. immitis*. The first and second, HS 3-13 and HS3-14, were located across the Kern River from Sharktooth Ridge in Oildale, California. They both yielded the same genotype (FIG. 2). The third

sample, EPS 7A from Elkhorn Plateau, yielded a different genotype. Of the 45 soil samples obtained in 1994, only one, J 10 from Elkhorn Plateau, yielded isolates of *C. immitis*. Two distinct genotypes were obtained from this soil sample, differing at four of the eight microsatellite loci. None of the 140 soil samples obtained in 1995 were positive for *C. immitis* using this screening method.

There was considerable variation in the numbers of colonies obtained per gram of soil. They ranged from less than one viable arthroconidium or colony forming unit (CFU)/gram of soil for EPS-7A to over 10 for HS 3-14. In total, DNA from 16 isolates was obtained. They represented 4 distinct genotypes and cultures were obtained for 11 of the 16 and included all 4 genotypes. Cultures were not obtained for all 16 samples since 5 of the subcultures prepared for the culture collection were overgrown with competing fungi. DNA extraction was performed on a portion of a single colony from a plate containing that colony and several colonies of other endemic fungi. The remainder of that colony was subcultured for archival purposes. Due to the presence of these other endemic fungi, often actively producing ascospores, combined with the poor competitiveness of *C. immitis*, subculturing was not always successful.

DISCUSSION

In keeping with what was known about *C. immitis*, this study found that its distribution is indeed sporadic and very limited. Of 720 soil samples screened, only 4 were positive for *C. immitis*. This less than 1-% recovery rate is within the range of documented recovery rates from earlier studies, but is still very low. The sampling criteria utilized (Swatek pers comm, Egeberg and Ely 1956, Elconin et al 1964) has been successful in isolating *C. immitis* from the environment. The isolation method utilized in this study (Omieczynski and Swatek 1967) is reported to be comparable to mammalian isolation methods. However, the culture isolation method is not as optimal for purification of cultures as is the utilization of a mammalian host. Of all the fungal species known to inhabit the endemic zone, only *C. immitis* produces spherules in an infected animal and only *C. immitis* can grow within host tissues. It is feasible that given the range of fungi inhabiting the endemic area, a mammalian host could be the most sensitive and specific selection vehicle available, though only able to select for isolates with a threshold virulence.

Climatic changes, seasonal variation and weather may also play a role in growth and thus retrieval of *C. immitis*. It has been noted in previous studies that the best time for retrieval of *C. immitis* from the environ-

ment is in the spring, within a few weeks of the last rains (Egeberg et al 1964, Elconin et al 1964, Kemp 1974). But as noted earlier, there is year to year variation in retrieval and there is also considerable variation year to year in cases. What factors, whether microclimatic changes to influx of susceptible individuals to changes in land usage and building patterns, that may cause this variation have yet to be determined.

The ecology and life cycle of *C. immitis* is to a large extent unknown. Only recently, by utilizing nucleic acid variation and genetic markers, has the fungus been definitively classified and genetic recombination shown (Bowman et al 1996, Burt et al 1996). The utilization of genetic markers has shown that *C. immitis* exists as two genetically isolated taxa (Burt et al 1997, Koufopanou et al 1998). The environmental isolates obtained were all of the California taxon. Repeated analyses of the same microsatellite locus in the same individual showed a variation of ± 0.5 bp. Therefore, in this study only allele differences greater than two base pairs, the length of a complete microsatellite repeat, were considered different alleles. Using these isolates and more geographically diverse isolates will determine to what extent these genetic markers developed for population and phylogenetic analysis can be used in epidemiological studies. Since the organism is not strictly clonal, a set of genotypes could demarcate a particular geographic location.

Given the mobility of humans, such geographical delineation is not possible from clinical samples except on a broad geographical level. There may be finer geographical groupings that are not readily apparent and which may be revealed in environmental samples of known geographic location and from clinical samples in outbreak investigations. Further analyses need to be done to determine if genotypes within one soil sample are more closely related than those from soil samples separated by several meters or even hundreds of kilometers. In addition to assessing the usefulness of these markers as epidemiological tools, such data could provide an idea of the amount and geographic extent of recombination.

Preliminary studies are currently being carried out to assess the virulence of environmental isolates obtained thus far. Since environmental samples may represent a wider virulence spectrum than isolates obtained from clinical samples, they may shed some light on the differences in disease severity that is seen with coccidioidomycosis.

ACKNOWLEDGMENTS

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