

Rev. Inst. Med. Trop. Sao Paulo  
55(1):7-11, January-February, 2013  
doi: 10.1590/S0036-46652013000100002

## VIABILITY AND MOLECULAR AUTHENTICATION OF *Coccidioides* spp. ISOLATES FROM THE INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO CULTURE COLLECTION, BRAZIL

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### SUMMARY

Coccidioidomycosis is an emerging fungal disease in Brazil; adequate maintenance and authentication of *Coccidioides* isolates are essential for research into genetic diversity of the environmental organisms, as well as for understanding the human disease. Seventeen *Coccidioides* isolates maintained under mineral oil since 1975 in the Instituto de Medicina Tropical de São Paulo (IMTSP) culture collection, Brazil, were evaluated with respect to their viability, morphological characteristics and genetic features in order to authenticate these fungal cultures. Only five isolates were viable after almost 30 years, showing typical morphological characteristics, and sequencing analysis using Coi-F and Coi-R primers revealed 99% identity with *Coccidioides* genera. These five isolates were then preserved in liquid nitrogen and sterile water, and remained viable after two years of storage under these conditions, maintaining the same features.

**KEYWORDS:** Culture collection; *Coccidioides*; Viability; Molecular identification; Long-term preservation.

### INTRODUCTION

*Coccidioides* spp. is a dimorphic fungus that causes coccidioidomycosis, a systemic mycosis affecting humans and a wide variety of animals. The disease is acquired through inhalation of infective arthroconidia that propagates in soil where the fungus lives in the mycelial form. Coccidioidomycosis is endemic in the semi-arid regions of southwestern United States, northern Mexico, and certain areas of Central and South America. It is considered to be a re-emerging disease in the United States, as the number of cases has increased markedly over the past decade<sup>8</sup>.

Since 1978, the disease has been diagnosed in several patients in the semi-arid northeastern region of Brazil, more frequently in Piauí, Ceará, Maranhão, and Bahia states<sup>10,14,23,31,33</sup>. Studies using delayed hypersensitivity tests with coccidioidin have demonstrated the presence of healthy infected individuals in these states, which are now considered to be endemic areas in Brazil<sup>6,7,33</sup>. It is possible that the disease remains underdiagnosed and that its geographic distribution extends throughout the northeastern region of the country<sup>11</sup>.

Diagnosis of coccidioidomycosis is based on culture isolation, serology, and histopathology. The identification of the fungus requires *in vitro* conversion of the mycelial form into the parasitic phase and/or the use of molecular probes<sup>2,5,9,27</sup>. Because handling of *Coccidioides* spp. is dangerous, many researchers have developed molecular techniques to

diagnose the disease. Polymerase chain reaction (PCR) assays targeting conserved or variable regions of the ribosomal DNA to detect and identify *Coccidioides* spp. have been described and found to be sensitive and specific<sup>2,12,15,16,18,21,27,30,32</sup>.

Molecular phylogenetic methods based on single-nucleotide polymorphisms and microsatellite repeats have established a new species, *Coccidioides posadasii*, which is morphologically indistinguishable from *C. immitis*<sup>12,19,30</sup>. Differences in the amino acid composition of various proteins between the two species suggest that other phenotypic differences may exist, including differences in factors involved in pathogenicity<sup>12</sup>.

The preservation of pathogenic fungi is essential for systematics, epidemiology, pathogenesis and genetic studies. The methods used for preservation should allow high-quality, long-term storage and full recovery, as well as ensuring phenotypic and genetic integrity of the organisms<sup>25,26</sup>. Subculturing can damage isolates, resulting in a decrease in spore production and pleomorphism, in addition to increasing the risk of contamination of samples<sup>4,22</sup>. There are several other methodologies available for the preservation of fungi that present various advantages and disadvantages, depending on the fungus group in question<sup>4,26,29</sup>.

The two techniques most widely used by culture collections for long-term preservation are cryopreservation in liquid nitrogen and freeze-drying, both of which maintain phenotypic and genotypic features of

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the isolates and prevent contamination. However, no single method can be applied generically to all fungi. For instance, although both sterile water and mineral oil can be used for preservation of filamentous and dimorphic fungi for several years<sup>25</sup>, factors such as metabolism, age of the inoculums and quality of the mineral oil influence the ability of the fungus to survive under mineral oil<sup>1,20</sup>. However, as *Coccidioides* spp. pose a high risk to laboratory personnel, the organisms should be preserved under non-culturing conditions<sup>11,32</sup>.

With the implementation of biological resources centers, organisms from culture collections have been increasingly used in the development of research projects, industrial processes and training courses. It has therefore become necessary to develop better methods of preservation suitable for different demands such as the amount of fungi (e.g., industrial processing or molecular studies) and the fungus form (mold or yeast), among other purposes, ensuring purity, reproducibility and authenticity.

The aim of this study was to analyze the viability, phenotypic and genotypic features of *Coccidioides* spp. isolates that have been maintained under mineral oil since 1975 in the IMTSP culture collection.

## MATERIAL AND METHODS

**1. *Coccidioides* spp. isolates:** The viability, morphological features and molecular characteristics of 17 isolates that were maintained in the Instituto de Medicina Tropical de São Paulo culture collection were evaluated. These

isolates were originally provided by research institutions in the USA and have been preserved under mineral oil since 1975 (Table 1).

**2. Fungal recovery:** Fragments of the 17 *Coccidioides* spp. cultures maintained under mineral oil were plated on Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and tryptic soy broth (TSB), and incubated at 30 °C for up to 60 days at room temperature to evaluate fungal recovery.

**3. Morphological characteristics:** The cultures grown on PDA medium were evaluated for colony morphology and for microscopy of typical arthroconidia. A fragment of culture was collected and placed on a slide with one drop of lactophenol cotton blue for microscopic examination.

**4. DNA isolation and sequence analysis:** Fragments of the viable isolates were cultured in 50 mL Brain Heart Infusion (BHI) broth, in a shaking incubator at 30 °C for 48 hours. In order to minimize the risk of infection, the cultures were submitted to boiling for 20 minutes. Thereafter, samples of each isolate were plated on SDA in slant tubes and incubated at 30 °C for four weeks. Samples were considered non-viable only when no growth occurred after this time. All these procedures were performed in a safety cabinet. Two hundred milligrams of dry mycelia were submitted to freezing in liquid nitrogen, after which DNA isolation was performed using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturers' instructions.

**Table 1**  
*Coccidioides* isolates preserved under mineral oil in the IMTSP culture collection since 1975

Isolate Number	Isolation year	Origin	Year of preservation under mineral oil	Molecular Identification (sequencing)	Viability
IMTSP Ci26	*	USA	1980	-	Not viable
IMTSP Ci28	*	USA	1980	-	Not viable
IMTSP Ci29	*	*	1975	-	Not viable
IMTSP Ci213	1930	USA	1980	-	Not viable
IMTSP Ci248	*	*	*	-	Not viable
IMTSP Ci249	1930	USA	1979	-	Not viable
IMTSP Ci251	1963	Guatemala	1975	-	Not viable
IMTSP Ci252	*	USA	1980	<i>C. immitis</i>	Viable
IMTSP Ci268	*	*	*	-	Not viable
IMTSP Ci332	*	*	*	-	Not viable
IMTSP Ci334	*	*	1976	-	Not viable
IMTSP Ci342	1930	*	1980	-	Not viable
IMTSP Ci397	*	*	1982	-	Not viable
IMTSP Ci485	1958	USA	1979	<i>C. immitis</i>	Viable
IMTSP Ci510	1945	USA	1980	<i>C. immitis</i>	Viable
IMTSP Ci520	1945	USA	1978	<i>C. posadasii</i>	Viable
IMTSP Ci604	1939	USA	1980	<i>C. posadasii</i>	Viable

\*unknown

DNA samples were first submitted to PCR amplification with ITS1 and ITS4 primers<sup>13</sup> in order to verify DNA integrity and the absence of amplification inhibitors. Once this had been established, 10 ng of the DNA samples were used as a template in PCR assays employing *Coccidioides*-specific primers CoiF (5'-TACGGTGTAATCCCGATAACA-3') and CoiR (5'-GGTCTGAATGATCTGACGCA-3') to amplify a 720-bp amplicon of *C. immitis*, as previously described by UMEYAMA *et al.* (2006). Amplifications were performed in 25 µL reaction volumes consisting of 2.5 U *Platinum Taq* polymerase (Invitrogen, Brazil), 1.5 mM MgCl<sub>2</sub>, 200 µM deoxynucleotide triphosphates (Invitrogen) and 10 pmol of each primer in 1× enzyme buffer (Invitrogen). Reaction mixtures were submitted to the following conditions: one cycle at 94 °C for three minutes, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and a final extension step at 72 °C for seven minutes.

Two microliters of each PCR product were submitted to electrophoresis in 2% ethidium bromide-stained agarose gels and visualized in a photodocumentation system (Vilber Lourmat, France). The ITS amplified products were purified using the PureLink™ PCR purification kit (Invitrogen), and sequenced in both forward and reverse directions using an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, USA). The sequences obtained were edited and aligned by means of the BLAST program (GenBank Database).

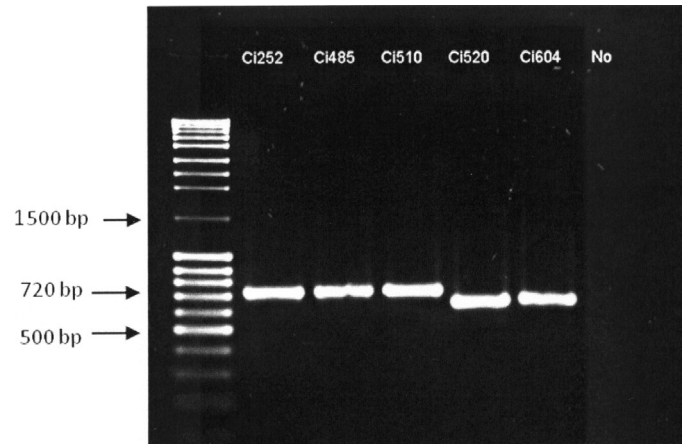
#### 5. Preservation of isolates in sterile water and liquid nitrogen:

Samples of the viable and authenticated *C. immitis* and *C. posadasii* isolates were then maintained in sterile water and liquid nitrogen for further viability evaluation. For this purpose, fragments of cultures grown on the PDA medium were collected and transferred to vials containing 5 mL of sterile water and stored at 4 °C, while other fragments were placed in cryovials containing glycerol as the cryoprotectant and maintained in the vapor phase in liquid nitrogen storage tanks. After two years of maintenance under these conditions, the organisms were cultured in PDA again to assess recovery.

### RESULTS

Only five of the 17 isolates maintained under mineral oil for almost 30 years in the IMTSP culture collection were viable after 60 days of incubation in the three media employed for this purpose. These five isolates of *Coccidioides* spp. were also all recovered after two years of storage in liquid nitrogen and sterile water, and the morphological characteristics of the genera were retained. These isolates presented typical morphological characteristics of *Coccidioides* such as white velvety colonies, hyaline and septate hyphae, although only two isolates (Ci252 and Ci485) revealed typical arthroconidia. These methods employed therefore increase the chances of retaining viability and morphological and genetic stability, and can be applied to long-term preservation of dimorphic fungi<sup>4,17,24</sup>.

The results of PCR employing the *Coccidioides*-specific primers (CoiF and CoiR) revealed that two of the five *Coccidioides* isolates (Ci520 and Ci604) were *C. posadasii* species. The amplified product from this species is shorter than that from *C. immitis*, revealing a deletion of nucleotides in the corresponding sequence (Fig. 1). Our results corresponded to the findings of UMEYAMA *et al.* (2006), who had demonstrated that these primers could distinguish the two *Coccidioides* species by comparison of the amplicon sizes on agarose gels.



**Fig. 1** - Agarose gel (2%) showing PCR products after amplification with CoiF and CoiR primers (UMEYAMA *et al.*, 2006). Ci252/Ci485/Ci510 correspond to *C. immitis* (720 bp), and Ci520/Ci604 correspond to *C. posadasii*. No: negative control. MW: 100 bp molecular weight marker (MassRuler DNA ladder, Fermentas, USA). The amplified product is smaller in *C. posadasii* than in *C. immitis*, reflecting deletion in the corresponding DNA sequence.

The DNA sequences of the amplicons obtained with the ITS primers were compared with the *Coccidioides* reference strain sequences available in GenBank. Two isolates (Ci520 and Ci604) showed 99-100% identity with *C. posadasii* (accession number AB2329900) and the other three isolates (Ci252, Ci435 and Ci510) showed 99% identity with *C. immitis* sequences (accession number AB232890).

### DISCUSSION

Since coccidioidomycosis is an emerging fungal disease in Brazil, the adequate maintenance and authentication of *Coccidioides* isolates are essential for research into genetic diversity of the environmental organisms, as well as for understanding the human disease. We showed that of the 17 isolates kept under mineral oil for almost 30 years at the IMTSP culture collection, only five remained viable. The low viability of the isolates may be related to the preservation and storage conditions. The mineral oil method is based on the reduction of the metabolic rate of the microorganism due to decreased oxygen consumption, which possibly affects conidiogenesis and viability, especially in fungi such as dimorphic fungi which have stringent metabolism<sup>1</sup>. High rates of viability using this preservation method have mainly been reported for filamentous fungi; this is probably because dimorphic fungi are more susceptible to the accumulation of toxic metabolites that may occur during the process of metabolic rate reduction, causing a decrease in viable fungus recovery<sup>22</sup>. In addition, the protracted period of almost 30 years for which the isolates remained under mineral oil may have affected the survival of the fungi<sup>3</sup>. Additionally, other factors such as the quality of the mineral oil and variability in the storage temperature may have affected the survival rates, as has previously been observed by other authors<sup>20,28</sup>. In addition, while storage in liquid nitrogen is a well-established storage method for many fungi, no single method should be used for preservation as some methods are not adequate for all organisms. Accordingly, international guidelines recommend that a second method should be used, along with multiple sampling for each isolate<sup>34</sup>.

Safe extraction procedures are essential when handling cultures of

hazardous fungal pathogens. The inactivation of *Coccidioides* cultures by heat was required to minimize the risk of infection; boiling the cultures was effective for inhibiting growth while not affecting the quality of the extracted DNA<sup>15</sup>. The use of PCR assays for identification of *Coccidioides* reduces the time for analysis, decreases manipulation of hazardous material and provides reliable data, enabling authentication of the isolates<sup>2</sup>.

Molecular approaches have revealed many fungal species that were previously unknown. However, in addition to the genotypic analysis, species identification should include a description of the morphological features, physiological characteristics and biochemical features, thereby building up the polyphasic taxonomy and ensuring the authenticity of the microorganisms maintained in culture collections, and in this way reliably providing verified organisms for research.

## RESUMO

### Viabilidade e autenticação molecular de isolados de *Coccidioides* spp. da coleção de culturas do Instituto de Medicina Tropical de São Paulo, Brasil

Coccidioidomicose é uma doença emergente no Brasil; a manutenção adequada e autenticação de isolados de *Coccidioides* spp são essenciais para a pesquisa em diversidade genética de micro-organismos, bem como para a compreensão da doença em humanos. Dezessete isolados de *Coccidioides* preservados em óleo mineral desde 1975 na coleção de culturas do Instituto de Medicina Tropical de São Paulo (IMTSP) foram avaliados com relação à viabilidade, características morfológicas e genéticas, com o objetivo de autenticação das culturas fúngicas. Dos 17 isolados, apenas cinco foram viáveis após quase 30 anos mantidos em óleo mineral, apresentando características morfológicas e moleculares típicas do gênero, o sequenciamento utilizando os oligonucleotídeos Coi-F e Coi-R revelou identidade de 99% com isolados de *Coccidioides*. Estes cinco isolados foram preservados em nitrogênio líquido e água destilada esterilizada, e permaneceram viáveis após dois anos de armazenamento sob estas condições, mantendo as mesmas características.

## ACKNOWLEDGMENTS

This work was supported by FAPESP (Process: 2007/55958-3)

## CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Received: 6 January 2012

Accepted: 1 October 2012