ARTIGO/ARTICLE

Viability and molecular authentication of *Coccidioides immitis* strains from Culture Collection of the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

Viabilidade e autenticação molecular de cepas de *Coccidioides immitis* da Coleção de Culturas do Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

Claudia C.F. Bezerra[1], Renata F. de Lima[2], Márcia S. Lazera[1], Bodo Wanke[1] and Cíntia M. Borba[2]

ABSTRACT

Twenty Coccidioides immitis *strains were evaluated. Only 5 of the 20 strains kept under mineral oil maintained their viability while all 5 subcultures preserved in water remained viable and none of the 13 subcultures kept in soil were viable. A 519 bp PCR product from the* csa *gene confirmed the identity of the strains.*

Key-words: Coccidioides immitis. *Long-term preservation. PCR.*

RESUMO

Vinte cepas de Coccidioides immitis *foram avaliadas. Cinco das 20 cepas preservadas sob óleo mineral mantiveram-se viáveis, todas as 5 subculturas preservadas em água permaneceram viáveis e nenhuma das 13 subculturas mantidas em solo foi viável. Um produto de PCR de 519 pb do gene* csa *confirmou a identidade das cepas.*

Palavras-chaves: Coccidioides immitis. *Preservação por longos períodos. PCR.*

Coccidioides immitis is a dimorphic, geophilic fungus that causes coccidioidomycosis, a systemic mycosis affecting humans and a wide variety of animals. This mycosis is endemic in many countries of the American continent. The highest prevalence of infection occurs in the Southwestern states of the USA and the Northern Mexican states. However, endemic foci are known in Central and South America. More recently, a large semiarid area in the Northeastern region of Brazil was identified as endemic for this mycosis[11 23].

Twenty *C. immitis* strains are maintained in the Culture Collection of the Oswaldo Cruz Institute using different long term storage methods. They were deposited in the collection between 1929 and 1955 and their records show that they came from the USA (9), Venezuela (4), Brazil (1), Netherlands (1), Norway (1) and Argentina (4). Fisher et al[13] described the division of *Coccidioides immitis* into two species, *C. immitis* and *C. posadasii*, respectively the agents of coccidioidomycosis in California and non-California population. Thus, despite the fact that *C. posadasii* is the agent of mycosis in Brazil, we decided to maintain the older taxonomic status due to the earlier isolation of the strains.

Fungi preservation methods have been used in many laboratories with differing results, depending on the species[1 12 16 24]. However, some studies have demonstrated that the storage of fungi *in vitro* for long periods of time may induce morphological changes[22], alterations in cell wall components[21], spontaneous mutations[3] and loss or attenuation of virulence[4]. The preservation of fungi which are pathogenic to man and animals is important for research and biotechnology, thus, determining the correct preservation method for each fungal species and periodical monitoring to check their identity has become a requirement. Molecular

1. Serviço de Micologia do Instituto de Pesquisa Clínica Evandro Chagas da Fundação Oswaldo Cruz, Rio de Janeiro, RJ. 2. Departamento de Micologia do Instituto Oswaldo Cruz da Fundação Oswaldo Cruz, Rio de Janeiro, RJ.

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Address to: Dra. Cintia de Moraes Borba. Depto. de Micologia IOC/FIOCRUZ. Av. Brasil 4365, 21040-900, Rio de Janeiro, RJ, Brasil.
Tel: 55 21 3865-8114; Fax: 55 21 3865-8113.
e-mail: cborba@ioc.fiocruz.br

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techniques have proved to be useful for specimen authentication and for obtaining molecular genetic data.

C. immitis produces a specific 19-kDa antigen encoded by the *csa* gene, with no cross-reaction with other fungi. The primer pair derived from this gene amplify a 519 bp product only when the *C. immitis* genomic DNA is used as template[17].

The objective of the present study was to evaluate the viability and morphological characteristics of *C. immitis* strains maintained for many years under mineral oil, in soil and in water from the Culture Collection of the Oswaldo Cruz Institute (IOC) in Rio de Janeiro, Brazil, and to authenticate them by PCR.

MATERIAL AND METHODS

A total of 20 *Coccidioides immitis* strains were first preserved at the IOC by successive transplants and maintained at room temperature. In the 1940's the strains were transferred to potato dextrose agar (PDA) medium under mineral oil. Each strain preserved under mineral oil was distributed among a maximum of 5 tubes with different dates, whereas each of the strains preserved in soil and water was represented by only one tube. Samples of the strains subjected to the different preservation methods were grown on PDA (Difco Laboratories, MI, USA) and incubated for 90 days at room temperature to evaluate viability. Figure 1 shows the procedure followed to extract the strains from the preservation methods and their evaluation.

A fragment of the colony grown on PDA was removed and then placed on a slide with one drop of Amann lactophenol – cotton blue (20g of phenic acid, 20ml of lactic acid, 20ml of distilled water, 40ml of glycerol and 0.05g of cotton blue) and covered with a coverslip for examination with a Nikon model Labophot microscope.

The viable strains were cultured in 100ml GYE medium (2% glucose, 1% yeast extract, all from Difco) and incubated at 30ºC in a reciprocating shaker at 120 oscillations/min. After 9 days of growth, the strains were subjected to 100ºC in order to kill them. The mycelium mat was separated by vacuum filtration using a sterile filter paper, followed by continuous vacuum until dry. The mycelium mat was lyophilized overnight in a freeze dryer, model L4KR 156 (Edwards, São Paulo, Brazil). Ten milligrams of the dried mycelia were grounded in liquid nitrogen. The resulting mycelium powder was suspended in 400ml of extraction buffer (50mM Tris-HCl pH 8.0; 100mM NaCl; 5mM EDTA and 1% SDS) incubated at 80ºC for 10 min, and incubated again at 40ºC with 10ml of proteinase K (10mg/ml) for 3h. Afterwards, the solution was incubated at 80ºC for 10 min in accordance with Burt et al[7]. The nucleic acids were then immediately extracted with 400ml of phenol: chloroform: isoamyl alcohol (25:24:1) and briefly homogenized. The solution was centrifuged at 10,000 x g for 15min. The upper aqueous phase was removed and the DNA precipitated with 3M sodium acetate and isopropanol. After washing with 70% cold ethanol the DNA was dried by continuous vacuum. The DNA was quantified in a spectrophotometer (GeneQuant™ *pro* RNA/DNA Calculator, Amersham Pharmacia, Biotech, USA) using 1:1,000 dilutions and visualized on 0.8% agarose gel.

Five nanograms of DNA were used as a template in PCR using specific primers designed on the basis of the *csa* gene sequence (sense = 5' AAG TTC TCA CTC CTC AGC GCT ATC G 3'; anti-sense = 5' ACA TTA AGG TTC CTC CCC TTC AAC C 3')[17]. The reaction mixture contained 10x buffer PCR (Amersham Pharmacia), 200mM of dNTPs, 10mM of each primer, 1 unit of Taq polymerase (Gibco), DNA template in a final volume of 50ml. Thirty cycles were conducted for amplification in a thermocycler (Gene Amp® PCR System 2,400 – Applied Biosystems). The amplification program was 1 cycle of 94ºC for 4 min and 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 74ºC for 1 min, followed by 74ºC for 10 min. After thermal cycling, 20ml of the amplified product was run on a 1.6% (w:v) agarose gel, with 1x TBE as buffer, stained with ethidium bromide and visualized under UV light.

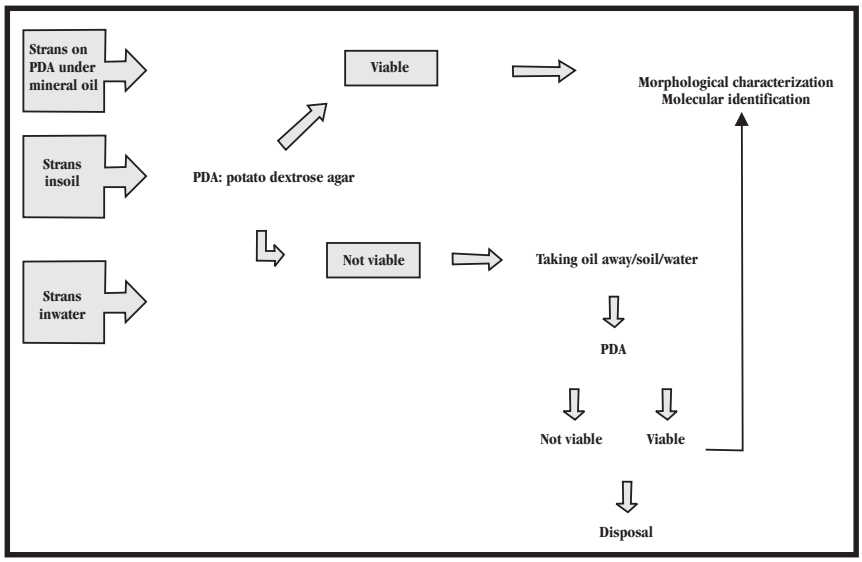

Figure 1 - Schematic representation of the evaluation of viability of* Coccidioides immitis *strains preserved in a culture collection.

RESULTS AND DISCUSSION

Only 5 (25%) of the 20 *C. immitis* strains maintained under oil remained viable, whereas all 5 (100%) strains preserved in water were viable and none of the 13 subcultures kept in soil were revealed as viable. Table 1 shows the data concerning the viable strains preserved over different periods of time.

Table 1 - C. immitis viable strains preserved using three preservation methods for different periods of time.

Strain number	Origin	Entry date	Preservation year/method	Oil depth (cm)	Viability
1294	USA	1929	1966/O	1.0	-
			1962/O	1.0	-
			1957/O	1.5	-
			u/O	1.2	+
			u/S	-	-
2171	Argentina	1947	1959/O	1.5	-
			u/O	1.3	+
			u/O	1.0	+
			u/S	-	-
2710	Argentina	1949	1953/O	d	-
			u/O	d	-
			u/O	d	-
			u/W	-	+
			u/S	-	-
2761	USA	1949	1949/O	0.1	-
			u/O	2.0	+
			u/O	3.0	-
			u/O	2.5	-
			u/W	-	+
			u/S	-	-
2762	USA	1949	1957/O	1.3	-
			u/O	2.0	+
			u/O	0.1	-
			u/S	-	-
2763	USA	1949	1957/O	1.0	-
			u/O	3.0	-
			u/O	3.0	ct
			u/W	-	+
			u/S	-	-
2764	USA	1949	1959/O	1.0	-
			u/O	0.1	-
			u/O	2.0	+
			u/O	2.0	-
			u/W	-	+
			u/S	-	-
2765	USA	1949	1977/O	0.1	-
			1977/O	0.5	+
			1972/O	0.1	-
			1953/O	1.0	-
			1953/O	0.5	-
			u/W	-	+

u = unknown; ct = contaminated; d = dry; O = mineral oil; W = water; S = soil

The *C. immitis* strains studied here were kept for long periods of time under mineral oil. This preservation method is based on the reduction of microorganism metabolic rates due to reduced oxygen consumption[6]. However, this decreased metabolic activity over long periods of time may affect the viability, development, spore formation and dimorphic process of many fungal strains[2 14 22]. Other determinants of the fall in viability are the depth of the mineral oil layer and the quality of the product used[10 19]. The small percentage of viable *C. immitis* strains after preservation under mineral oil detected in this study may be related to their intrinsic characteristics and also to variations in the attributes of the preserved cells and of the technique used. Thus, the low viability of the strains studied clearly shows the high requirements of this fungus for factors related to good development *in vitro*.

In contrast, the *C. immitis* cells evaluated here that were preserved in distilled water remained viable for 36 years. Water storage is a simple and inexpensive method of culture preservation described by Castellani[8], who stored fungi pathogenic to man. Of the fungi studied by Castellani[9], *C immitis* grew very well, producing colonies exactly like the original, with the same biochemical characters after 12 months in sterile distilled water. McGinnis et al[15] obtained 93% revival for 12-60 months in cultures preserved in distilled water and recorded reduced pleomorphism in pathogens.

Many of the fungal pathogens of humans accommodated in the Onygenales, like *C. immitis*, including agents of cutaneous infection (*Microsporum* and *Trichophyton*) and human respiratory pathogens (*Histoplasma* and *Blastomyces*), showed a rate of viability ranging between 73% and 100% after 6, 12, 18 and 24 months in sterile distilled water[20]. These data are in agreement with our results which show 100% viability for *C. immitis* strains preserved in distilled water for a long period of time (36 years). According to Onions[16] this method appears to produce very satisfactory results for some isolates.

None of the strains studied survived when preserved in soil, in agreement with Windels[24], who reported that the use of soil culture for long-term storage may cause mutations with the loss of morpho- and physiological characteristics or cellular death.

The viable strains presented macro and microscopic aspects suggestive of *C. immitis* as white cream colored velvety colonies formed by hyaline, septate, branched hyphae and only one strain (Ci IOC 2761 preserved in water) presented arthroconidia alternating with empty cells. However, in accordance with Pappagianis[18] the hyphal form is not sufficiently specific for that to yield more than a presumptive diagnosis. In order to avoid the animal inoculation method to demonstrate their virulence, these isolates were authenticated by a molecular tool (PCR). A 519bp PCR product was obtained with the primer pair used and *C. immitis* genomic DNA was used as the template to confirm the identity of the viable strains (Figure 2). The results of the PCR method for the detection of *C. immitis* DNA using oligonucleotide primer pair derived from *csa* gene sequence showed an amplification of identical 519bp product in all seven strains of

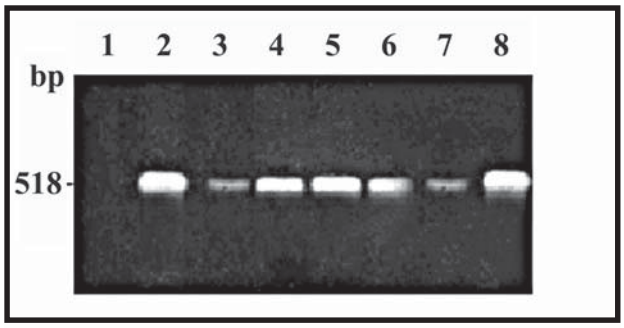

Figure 2 - Agarose gel showing PCR products obtained by amplification of 5 ng of DNA, extracted from the* C. immitis *strains. Lane 1: no template DNA; Lane 2: Ci IOC 1294; Lane 3: Ci IOC 2171; Lane 4: Ci IOC 2762; Lane 5: Ci IOC 2763; Lane 6: Ci IOC 2764; Lane 7: Ci IOC 2765; Lane 8: Ci IOC 2761. The amplification reactions are described in the text. Molecular weight markers (bp) are indicated on the left side.

the pathogen in accordance with Pan and Cole[17], demonstrating the usefulness of this molecular marker for culture collections.

Culture collections are an important resource for obtaining molecular genetic data and the PCR technique is an effective method for this. The problems of the correct classification of species names, alterations in morphological characteristics, which prevent precise identification and changes in population structure over time, can be examined by analysis of DNA regions. According to Bruns et al[5], extensive use of preserved specimens for molecular genetic investigations may soon make it desirable for curators to develop guidelines for the sampling of type collections and for methods of storage and retrieval of extracted DNA.

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