COMPARISON OF STORAGE METHODS TO PRESERVE THE PATHOGENIC OOMYCETE *PYTHIUM INSIDIOSUM*

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Abstract. *Pythium insidiosum* causes life-threatening pythiosis in humans and other animals. Study of this organism requires maintaining it in culture which can be difficult. The aim of this study was to compare several methods of maintaining *P. insidiosum* in cultures in order to determine the best method to accomplish this. We compared the following methods for maintaining *P. insidiosum*: routine repeated subculture, distilled water immersion, liquid paraffin overlay and storage at an ultra-low temperature with liquid nitrogen. Ribosomal deoxyribonucleic acid (rDNA) sequences of *P. insidiosum* were analyzed to confirm the identity of the organism after preservation. We conducted an initial 2-week assessment but due to relatively low viability with storage at an ultra-low temperature with liquid nitrogen, this method was not used for the long term study. The remaining methods were reassessed every 3 months for 15 months. The routine repeated subculture, distilled water immersion and liquid paraffin overlay methods showed the viability rates of 100%, 100% and 90% at 9 months, 100%, 90% and 80% at 12 months and 100%, 60% and 40% at 15 months, respectively. The rDNA sequences of the preserved strains showed no differences compared to the original strains. We conclude the distilled water immersion method is optimal due to being simple to perform, inexpensive and relatively efficient for maintaining *P. insidiosum* for at least 9 months. This method reduces the frequency of routine repeated subculture passages by 9 fold.

Keywords: *Pythium insidiosum*, pythiosis, preservation, storage, culture collection

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INTRODUCTION

Pythium insidiosum is an under-recognized potentially devastating pathogen. It is a member of oomycetes, aquatic microorganisms that belong to the Kingdom Stramenopiles (Kamoun, 2003). *P. insidiosum* can cause a potentially life-threatening infection, known as pythiosis, in humans and other animals and is reported predominately from tropical

and subtropical countries (Thianprasit *et al*, 1996; Krajaejun *et al*, 2006; Gaastra *et al*, 2010). Diagnosis and treatment of pythiosis require experience and often intensive medical care. Loss of infected organs, such as an eye or a leg or even death are common outcomes for infected patients (Krajaejun *et al*, 2006).

Multidisciplinary studies are needed to determine the biology, pathogenicity, epidemiology and clinicopathology of *P. insidiosum*; these can lead to better diagnosis and treatment.

Isolation of *P. insidiosum* from clinical specimens is relatively rare and difficult, compared to bacteria and fungi. It is important to maintain *P. insidiosum* in culture for prospective biological, epidemiological and clinical studies. However, maintaining a *P. insidiosum* culture is labor intensive and time consuming. A simple, efficient, cost-effective method for long-term maintenance of *P. insidiosum* is needed.

A number of methods for maintaining fungal cultures short-term (≤ 1 year) and long-term (> 1 year) have been described (Castellani, 1964; Hwang, 1966; Gale *et al*, 1975; de Capriles *et al*, 1989; Nishii and Nakagiri, 1991; Pasarell and McGinnis, 1992; Nakasone *et al*, 2004; Baskarathevan *et al*, 2009; Guimarães *et al*, 2014). Short-term preservation can be accomplished by periodically repeating subcultures, which is feasible for a small culture collection (Nakasone *et al*, 2004). Long-term preservation methods include sclerotization; oil overlay; immersion in distilled water; lyophilization; storage in an organic substance such as wood chips, cereal grains or agar strips; storage in silica gel for sporulating fungi; storage in a freezer or with liquid nitrogen (Castellani, 1964; Hwang, 1966; Gale *et al*, 1975; de Capriles *et al*, 1989; Nishii and Nakagiri, 1991; Pasarell and McGinnis, 1992; Nakasone *et al*, 2004; Baskarathevan *et al*, 2009; Guimarães *et al*, 2014). These methods require a variety of different equipment and supplies and degrees of skill at preserving the organism of interest for long periods of time, such as up to several decades. *P. insidiosum* does not sporulate and storage requires expensive equipment; therefore, typical preservation methods, such as sclerotization, storage at an ultra-low temperature and lyophilization, are impractical for routine clinical laboratories. Therefore, we aimed to determine the efficiency of alternative methods for preservation: monthly routine subculture, liquid paraffin overlay, distilled water immersion and storage with liquid nitrogen. The rDNA sequences of *P. insidiosum* were amplified and analyzed to ensure the identity of the organisms after preservation.

MATERIALS AND METHODS

Microorganisms and culture conditions

Five isolates of *P. insidiosum* from patients with pythiosis were used for this study: 3 isolates from patients with vascular pythiosis (strains Pi12, Pi31 and Pi35) and 2 strains from patients with ocular pythiosis (strains Pi20 and Pi25). The identity of each isolate was confirmed by zoospore induction (Chaiprasert *et al*, 1990) and rDNA sequence homology analysis (Badenoch *et al*, 2001). All isolates were subcultured monthly on 8 ml Sabouraud dextrose slant agar at a pH of 7.2 and a temperature of 28ºC contained in a 20 x 150 mm glass tube. To test each of the preservation methods, 5 mm diameter plugs were cut from the edge of a one-week-old colony of each *P. insidiosum* strain grown on 10 ml Sabouraud dextrose agar at a pH of 7.2 contained in a 90 mm diameter Petri dish.

Storage methods

The study was conducted for 2 time intervals: 2 weeks and 15 months. This was done to rapidly identify flawed storage methods. The methods tested during the 2-week study were: routine repeated subculture (see above), distilled water immersion, liquid paraffin overlay and storage with liquid nitrogen. After the initial 2-week study it was determined the best methods to conduct the 15-month study were: routine repeated subculture, distilled water immersion and liquid paraffin overlay. Each study was performed in duplicate. The methods used followed those described previously (Castellani, 1964; Hwang, 1966; Gale *et al*, 1975; de Capriles *et al*, 1989; Nishii and Nakagiri, 1991; Pasarell and McGinnis, 1992; Nakasone *et al*, 2004; Baskarathevan *et al*, 2009; Guimarães *et al*, 2014) as follows.

Distilled water immersion

Two agar plugs with actively growing hyphae from each *P. insidiosum* strain were transferred into a screw-cap glass tube (20 x 150 mm) containing 5 ml sterile distilled water. The cap was then placed on the tube which was then stored in an upright position at room temperature until testing.

Liquid paraffin overlay

A slant agar tube (20 x 150 mm) with an actively growing culture (aged 10 days) from each *P. insidiosum* strain was overlaid with 6 ml sterile liquid paraffin, to completely cover the colony surface. The cap was then placed on the tube which was then kept upright at room temperature until testing.

Liquid nitrogen storage

Two agar plugs with actively growing *P. insidiosum* hyphae were transferred to a 2-ml cryogenic tube (Corning, Acton, MA) containing 1 ml of 10% dimethyl sulfoxide (DMSO; as a cryoprotectant). The tube was placed in a freezer at -30ºC for 30 minutes and then transferred to a liquid nitrogen tank (MVE cryosystem 2000, Washington, PA) for separately storing in the vapor and liquid phases where it was kept until testing. The tube was thawed to room temperature and the DMSO was washed out with sterile water.

Evaluation of *P. insidiosum* viability

At the end of the 2-week study and every 3 months during the 15-month study, 2 agar plugs containing *P. insidiosum* hyphae were removed from each container, inoculated on a fresh Sabouraud dextrose agar plate and incubated at room temperature for one week. An organism was considered viable if growth was observed and non-viable if no colony appeared.

DNA extraction and sequencing

At the end of the 15-month study, viable organisms were examined for their genomic DNA (gDNA) using the salt-extraction method of Lohnoo *et al* (2014). The concentration and purity of the extracted gDNA were evaluated with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*, 1990) were used to amplify the rDNA sequence of the studied *P. insidiosum* in a 50-µl polymerase chain reaction (PCR) containing 100 ng of gDNA template, 5 pmole of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1.25 units of Taq polymerase and 1x Taq buffer with KCl (Thermo Scientific). PCR amplification was carried out in a Nexus Gradient Master Cycler (Eppendorf, Hamburg, Germany) using the following settings: 95ºC for 6 minutes; 25 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds; with a final step of 72ºC

for 10 minutes. The PCR product obtained was then examined with 1% agarose gel electrophoresis before purification with a Nucleospin Extract-II column (Macherey Nagel, Düren, Germany). The PCR product was then sequenced using the ITS1 and ITS4 primers, with a BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystem, Foster City, CA), and an ABI 3100 Genetic Analyzer (Applied Biosystem). Obtained sequences were searched against the National Center for Biotechnology Information (NCBI) nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Accession numbers of rDNA sequences

The rDNA sequences of the *P. insidiosum* studied strains (Pi12, Pi25, Pi31, Pi20 and Pi35) have been deposited in the NCBI database, under the accession numbers LC199876, AB898116, AB898120, AB971183 and AB898124, respectively.

RESULTS

Initial 2-week assessment of different storage methods

All the tested strains of *P. insidiosum* (100%) preserved by the routine repeated subculture and distilled water immersion methods survived the 2-week study period (Table 1). Seventy percent of the tested strains stored with liquid paraffin survived the 2-week study period (Table 1). Forty percent of the tested strains stored with liquid nitrogen survived the vapor phase of storage and none survived the liquid submerged phase of storage (Table 1).

15-month assessment of different storage methods

The storage methods evaluated during the 15-month assessment were: routine repeated subculture, distilled water immersion, and liquid paraffin overlay. The liquid nitrogen storage method was excluded because of unsatisfactory viability on the 2-week test. For the routine repeated subculture method, *P. insidiosum* colonies of each strain were plated on new Sabouraud dextrose agar every month. All the tested strains grew well with the routine repeated subculture and all (100%) survived the 15-month study. The distilled water immersion gave 100% viability for the first 9 months and the liquid paraffin overlay gave 100% viability for the first 6 months (Table 2). During the 15-month study period, with the distilled water storage method, evaporation gradually decreased the water levels of all tubes to about one-third the amount of the original level, except for one strain (Pi35) the water evaporated almost completely. The viability with the distilled water immersion method decreased from 100% at 9 months to 60% at 15 months (Table 2). With the liquid paraffin overlay method, the viability decreased from 100% at 6 months to 40% at 15 months (Table 2).

Identity of recovered organisms

On DNA sequencing at the end of the 15-month study, the DNA sequences of the isolates completely (100%) matched the sequence of the organisms prior to conducting the study.

DISCUSSION

Repeated subculture is a routine method for maintaining *P. insidiosum* in our laboratory and serves as a reference standard for comparing with the other methods examined. During our 2-week initial assessment the liquid nitrogen storage method resulted in poor viability (40% with the vapor phase and 0% with the liquid phase). Our findings are in contrast

Table 1
2-week viabilities of *Pythium insidiosum* study strains preserved using various methods. Duplicates of study strains are shown together. "+" represents growth, while "-" represents no growth.

Storage method	Strain					Number (%) with growth
	Pi12	Pi25	Pi31	Pi20	Pi35	
Subculture	+/+	+/+	+/+	+/+	+/+	10 of 10 (100%)
Distilled water	+/+	+/+	+/+	+/+	+/+	10 of 10 (100%)
Liquid paraffin	+/+	+/+	-/-	+/+	+/-	7 of 10 (70%)
Liquid nitrogen (vapor phase; -165 ºC)	+/-	+/-	-/-	+/+	-/-	4 of 10 (40%)
Liquid nitrogen (liquid phase; -196 ºC)	-/-	-/-	-/-	-/-	-/-	0 of 10 (0%)

Table 2
15-month viabilities of *Pythium insidiosum* study strains preserved using various methods. Duplicates of study strains are shown together. "+" represents growth, and "-" represents no growth.

Preservation method	Strain	Storage duration (months)					
		0	3	6	9	12	15
Subculture	Pi12	+/+	+/+	+/+	+/+	+/+	+/+
	Pi25	+/+	+/+	+/+	+/+	+/+	+/+
	Pi31	+/+	+/+	+/+	+/+	+/+	+/+
	Pi20	+/+	+/+	+/+	+/+	+/+	+/+
	Pi35	+/+	+/+	+/+	+/+	+/+	+/+
	Number (%) with growth	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)
Distilled water	Pi12	+/+	+/+	+/+	+/+	+/+	+/+
	Pi25	+/+	+/+	+/+	+/+	+/+	+/-
	Pi31	+/+	+/+	+/+	+/+	+/+	+/+
	Pi20	+/+	+/+	+/+	+/+	+/+	+/-
	Pi35	+/+	+/+	+/+	+/+	+/-	-/-
	Number (%) with growth	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)	10 of 10 (100%)	9 of 10 (90%)	6 of 10 (60%)
Liquid paraffin	Pi12	+/+	+/+	+/+	+/+	+/+	+/-
	Pi25	+/+	+/+	+/+	+/-	+/+	+/-
	Pi31	+/+	-/-	+/+	+/+	-/-	-/-
	Pi20	+/+	+/-	+/+	+/+	+/+	+/-
	Pi35	+/+	+/+	+/+	+/+	+/+	+/-
	Number (%) with growth	10 of 10 (100%)	7 of 10 (70%)	10 of 10 (100%)	9 of 10 (90%)	8 of 10 (80%)	4 of 10 (40%)

to those of Hwang (1966) who successfully preserved 10 different *Pythium* species in the nitrogen vapor phase (~165ºC) for 12 to 38 months. Nishii and Nakagiri (1991) successfully preserved 76 out of 79 isolates of 22 *Pythium* species in the nitrogen liquid phase (~196ºC) for as long as 2 years, regardless of the type of cryoprotectant (DMSO or glycerol) used. An important difference between our study and the above 2 studies that successfully preserved *Pythium* species with liquid nitrogen was the cooling rate. In their studies, they gradually cooled the isolates at a rate of -1ºC per minute until reached a temperature of -35 to -40ºC, before placing the storage tube in liquid nitrogen. The slow cooling reduces the likelihood of inducing injurious ice crystals (Nishii and Nakagiri, 1991) but we do not have access to this type of equipment, so the freezing in our study was nearly instantaneous and likely induced ice crystals.

Compared to the liquid paraffin overlay, the distilled water immersion gave a better viability rate at 9 months (100% *vs* 90%). A problem with the distilled water immersion method in our study is that the water evaporated and the drying out resulted in a decreased viability. However, Castellani (1964) found that fungi can be preserved for up to a year or longer if the evaporated water is replaced. de Capriles *et al* (1989) following a method similar to Castellani (1964) was able to preserve ~60% of all tested fungal strains ($n = 594$) from 116 different species for more than one year and some strains remained viable for up to 20 years. Replacing the evaporated water could improve long-term maintenance of *P. insidiosum* by the distilled water immersion method.

In conclusion, we found the distilled water immersion method was simple, inexpensive and adequate for preserving *P. insidiosum* for at least 9 months. This method reduced the need for the frequency of subculture passages by 9 fold. Our results suggest the distilled water immersion method is the best method for preserving *P. insidiosum* for up to 9 months due to its ease and cost, especially in resource-limited clinical and research laboratories.

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