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A Head-to-head Comparison of Four Cryopreservation Protocols of Dermatophyte Species

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Abstract

Aims: Transportation of clinical samples and long-term recoverability of fungal strains are critical to epidemiological studies. In addition, the study of fungi often requires the use of living pure cultures. The aim of this study was to evaluate the methods used to preserve culture collections of dermatophytes, consisted of storage in sterile distilled water, cryopreservation with glycerol, preserving in tryptic soy *broth* (TSB), and freezing mycobiotic agar.

Materials & Methods: in this experimental study, ninety-two dermatophyte isolates belonged to 10 species were tested. The freezing protocol was done in 4 forms of sterile distilled water, cryopreservation with glycerol, freezing mycobiotic agar, and preserving in TSB. The viability of the dermatophytes species was assessed after 3 years at morphological (macro and microscopic features), physiological (Using Dermatophyte Test *Medium;* DTM, urease test media, and the hair perforation test), and genetic levels by *restriction fragment length polymorphism* (*RFLP*).

Findings: The survival rate was 84 out of 92 water stored fungal strains (91.3%) and 81 out of 92 mycobiotic agar stored strains (88.0%) and 75 out of 92 glycerol 40% stored strains (81.5%) and 43 out of 92 TSB stored fungal strains (46.7%). Overall, more than 88% of the strains survived in the distilled water storage and freezing mycobiotic agar, methods, while storage in TSB had the least success in the maintenance of dermatophytes.

Conclusion: The procedure to preserve cultures in sterile distilled water is reliable, simple, and inexpensive.

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Dermatophytes [https://www.ncbi.nlm.nih.gov/mesh/68003883];
Cryopreservation [https://www.ncbi.nlm.nih.gov/mesh/68015925];
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Introduction

The study of fungi often requires the use of living pure cultures [1]. The traditional method used to preserve filamentous fungi is sub-culturing from staled to fresh media. Although this method is relatively inexpensive, it is time-consuming, prone to contamination, and requires physical space that makes it impractical for large-scale culture collections [1-3]. Moreover, it does not prevent the physiological and genetic variations during long-term maintenance [3, 4]. Thus, preservation methods were developed to overcome these limitations. The basis of these methods is to minimize the metabolism of microorganisms at low temperatures [1, 5]. These methods are:

1) Storage in sterile distilled water [6] or mineral oil [7] (Some strains may degenerate over time)
2) Lyophilization (Some strains can be long preserved) [6, 8]
3) Cryopreservation (Preservation temperature is generally at -80°C or at -196°C) [3, 9]
4) Preserving in Tryptic Soy Broth (TSB) [10]
5) Preservation of fungus stock cultures by deep-freezing [11]
6) Storage of dermatophytes in skin scales [12]

Because the stability of fungal cells at room temperature was not ensured by storage in sterile water, it has been suggested to store water vials at low temperatures (-20°C and -70°C) [13-15]. Also, the mineral oil covering which is easily contaminated is not suitable for the preservation of dermatophytes [6]. Lyophilization was introduced as an ideal method, but the procedure was cumbersome, lengthy, complicated, and required expensive equipment [6, 8, 15]. Currently, cryopreservation is a suitable method for preserving fungi [16]. In the cryopreservation method, microorganisms are suspended with a protective additive, then they are stored at -80 ^{0}C to -196 ^{0}C. Numerous factors affect the effectiveness of cryopreservation in microorganisms, e.g. species, cell size, form, growth phase, storage temperature, and composition of the medium used to suspend [4, 6, 8, 16, 17]. The presence of glycerol usually increases the survival considerably [15, 18]. The TSB is a liquid medium used to preserve microorganisms [10]. Altogether, the success of a preservation method depends on the compatibility of the protocols related to species and strain. Several previous studies of the genetic stability of cryopreserved fungi have been performed by *random amplification of polymorphic DNA* (RAPD) and Internal Transcribed Spacer (ITS) profiles [19, 20].

Dermatophytosis caused by filamentous fungi belongs to the following three genera: Microsporum, Trichophyton, and Epidermophyton [21]. Dermatophytes are known to be the most common cause of dermatomycoses and onychomycosis [22, 23]. In addition, the incidence of dermatophytosis and drug-resistant has been increasing over the last few decades [24].

The aim of this study was to evaluate the methods used to preserve culture collections of dermatophytes, consisted of storage in sterile distilled water, cryopreservation with glycerol, preserving in tryptic soy *broth (*TSB), and freezing mycobiotic agar.

Materials and Methods

This study is experimental.

Dermatophyte isolates: Ninety-two clinical dermatophyte isolates of 10 species obtained from different parts of Iran. *Epidermophyton floccosum* (n=18), *Microsporum canis* (n=4), *Microsporum gypseum* (n=8), *Microsporum ferrugineum* (n=2), *Trichophyton mentagrophytes* (n=20), *Trichophyton rubrum* (n=15), *Trichophyton verrucosum* (n=9), *Trichophyton tonsurans* (n=13), *Tricholosporum violaceum* (n=2) and *Trichophyton schoenleinii* (n=1) were cultured on two slants of Potato Dextrose Agar (PDA) per strain and incubated at 28°C for 2 weeks.

Storage of strains

Cryopreservation technique: Then sporulating cultures were homogenized in the first tube with physiological saline. To these prepared microbial suspensions was added protective cryopreservation medium (Sterile glycerol 40%) in the same amount as the saline [16, 17, 25]. These preparations were transferred to 1.5-ml microtubes, each vial labeled, the cap was tightened, and frozen to -20°C.

Storage on the TSB: A piece of the colony, including conidia and hyphae fragments of the second slants of PDA were transferred to 1.5-ml microtubes containing the TSB [10]. Then each vial labeled, the cap was tightened and stored at -20°C.

Freezing technique: Also, all specimens were cultured on mycobiotic agar slants in the microtubes 1.5-ml and incubated at 28°C for 2 weeks [11]. Each vial cap was tightened, labeled, and then stored at -20°C for 3 years.

Water culture technique: 4ml of sterile distilled water was added to the second tube of each culture. Then the conidia and hyphae fragments of the colonies were collected and transferred to sterile 1.5-ml microtubes. Then each vial labeled, the cap was tightened to prevent water evaporation, and stored at stored at -20°C [6, 26].

For culture survival assessment, 0.3ml of suspension from the frozen vials and a piece of the frozen colony into the microtubes were transferred to fresh mycobiotic agar or PDA slants and incubated at 28°C for 2 weeks. Then cultures were immediately returned to the freezer. In the case of growth paucity after 4 weeks, the isolates were recorded as not viable. Several tests were used to verify our identification with readings after 7 and 14 days such as urea hydrolysis, growth on Dermatophyte Test Medium (DTM) [25, 26] also, hair perforation test according to the method of Ajello [27].

DNA extraction: All fungal strains were cultured on mycobiotic agar (Merck; Germany) and incubated at 28°C for 4 days. DNA was extracted using the chloroform and proteinase K method. The fungal mycelium by pestle and mortar in addition to liquid nitrogen were crushed in DNA extraction buffer containing (200M Tris-HCL, pH=8, 25mM EDTA, SDS 0.5% W/V, NaCl 250mM). After vortexing, proteinase K was added and incubated at 55°C for 60min. The DNA was extracted with an equal volume of chloroform. Total nucleic acids were precipitated with isopropanol and washed in 300µL ethanol 70%, air dried, rehydrated in 50µl Tris-EDTA (TE) buffer, and stored at -20°C until it was used [28].

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) procedure: The ITS region was PCR amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [29]. Each mixture contained 12.5µl of premix, 1µl of DNA template, 0.3µM of each primer, and enough water to reach a final reaction volume of 25µl. Negative controls (Water instead of fungal DNA) were added to each PCR. The reaction mixture was initially denatured at 95°C for 5min, followed by 30 cycles of 30s at 94°C, 30s at 56°C, and 45s at 72°C, and a terminal extension step of 72°C for 5min. Five microliters of the PCR products were electrophoresed on 1% agarose gel in Tris-acetate-EDTA (TAE) buffer and then observed and visualized using ultraviolet irradiation.

All digestion reactions were performed in a 15µl mixture containing 2µl of 10×buffer, 2µl of the enzyme, 10µl of ITS amplicon, and enough ultrapure water up to the final volume. The amplified products were subjected to digestion with *MvaI* (Fermentas; United States) for 14h at 37°C [30]. PCR amplicons and restriction enzyme digestion products were loaded in 2.5% (W/V) agarose gels in the presence of a red gel (0.5µg.mL^{-1}). A DNA molecular weight marker, 100bp ladder (Fermentas; United States) was used (After running for 1.5h at 90V.cm^{-1}).

The sizes were expected from the region amplified by ITS and the restriction enzyme digestion with *MvaI* obtained from the website NEB cutter (http://tools.neb.com/NEBcutter).

Re-isolation procedure: After 3 years, the samples were cultured on mycobiotic agar and incubated at 25-28°C for up to 4 weeks. The viability, the morphological and genetic features of 10 dermatophytes species were examined. If the rate of growth, the morphology and the presenting color present were as the same of the original culture, each dermatophyte isolate was considered as viable [25, 26].

Findings

All the survived dermatophytes preserved their physiological characteristics, such as the production of urease, hair perforation, and the production of characteristic pigments on DTM (Table 1). The changes of the yellow color of DTM medium to red was seen in all isolates.

The results were different for storage at -20°C and were dependent on the preservative.

The survival rate was 84 out of 92 water stored fungal strains (91.3%) and 81 out of 92 mycobiotic agar stored strains (88.0%) and 75 out of 92 glycerol 40% stored strains (81.5%) and 43 out of 92 TSB stored fungal strains (46.7%). Overall, more than 88% of the strains survived in the distilled water storage and freezing mycobiotic agar, methods, while storage in TSB had the least success in the maintenance of dermatophytes. The lowest survival rate was observed in *T. schoenleinii* and highest rate *T. mentagrophytes*, followed by *T. tonsurans, T. rubrum,* and *M. gypseum*, respectively (Table 2).

Table 1) Morphological criteria for verifying species identification

Species Test	Hair perforation	Urea hydrolysis
T. mentagrophytes	+	+
T. rubrum	-	-
T. violaceum	-	-
M. canis	+	+
M. ferrugineum	-	+
T. schoenleinii	-	+
T. verrucosum	-	-
T. tonsurans	-	+
M. gypseum	+	+
E. floccosum	-	+

Table 2) Viability of cultures in distilled water, Mycobiotic agar, glycerol 40% and TSB after 3 years preservation (n=92)

Species	Water-stored culture Viable isolates/No. tested	Mycobiotic agar culture Viable isolates/No. tested	Glycerol 40% culture Viable isolates/No. tested	TSB culture Viable isolates/No. tested
E. floccosum	15/18	14/18	13/18	7/18
M. canis	4/4	4/4	3/4	1/4
M. gypseum	8/8	8/8	7/8	2/8
M. ferrugineum	1/2	1/2	1/2	0/2
T. mentagrophytes	20/20	20/20	18/20	13/20
T. rubrum	14/15	14/15	12/15	10/15
T. verrucosum	8/9	7/9	8/9	1/9
T. tonsurans	13/13	12/13	12/13	9/13
T. violaceum	1/2	1/2	1/2	0/2
T. schoenleinii	0/1	0/1	0/1	0/1
General survival rate (%)	84 (91.3)	81 (88.0)	75 (81.5)	43 (46.7)

According to the ITS-PCR result, the biggest size obtained was for *E. floccosum* and the smallest one for *M. gypseum.*

The obtained band sizes range from 14bp to 517bp among different species (Table 3).

All the specimen tested were identified at the level of species by the unique banding pattern to each species except for closely related species such as *M. canis*/*M. ferrugineum* showed the same profiles (Figure 1).

Table 3) The expected sizes of DNA fragments generated by enzymatic digestion *MvaI*

Dermatophyte species	DNA fragment (bp)
T. Mentagrophytes	247, 159, 124, 89, 50, 14
T. rubrum	368, 164, 95, 65
T. tonsurans	251, 124, 103, 90, 56, 50, 14
T. schoenleinii	405, 124, 104, 52
T. verrucosum	517, 141, 20
T. violaceum	396, 161, 106, 45, 20
M. ferrugineum	441, 165, 103, 28
M. gypseum	289, 179, 146, 33, 19
M. canis	441, 165, 103, 28
E. floccosum	361, 231, 169, 20

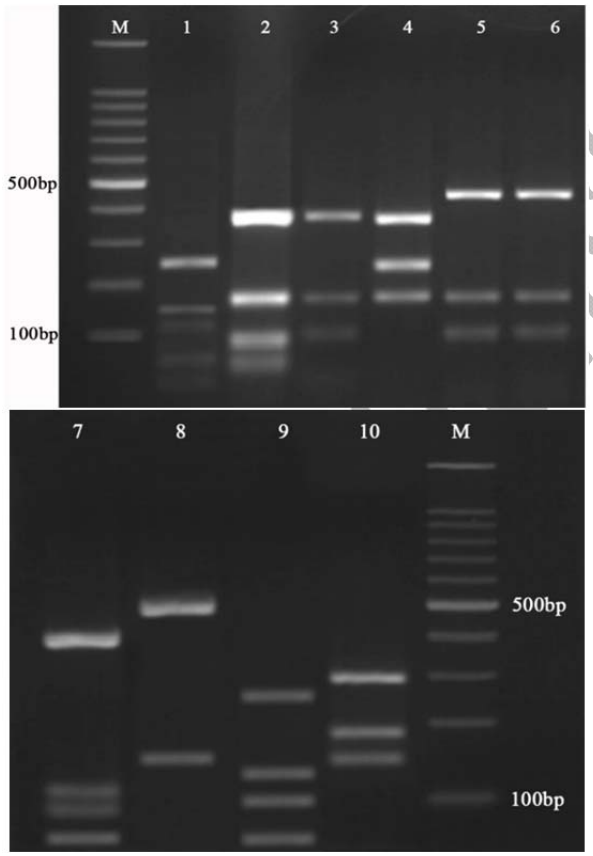

Figure 1) PCR-RFLP identification of dermatophytes at the level of species (**Lane M:** 100bp DNA ladder, **Lane 1:** *T. mentagrophytes*, **Lane 2:** *T. rubrum,* **Lane 3:** *T. violaceum*, **Lane 4:** *E. floccosum*, **Lane 5:** *M. canis*, **Lane 6:** *M. ferrugineum,* **Lane 7:** *T. schoenleinii*. **Lane 8:** *T. verrucosum*, **Lane 9:** *T. tonsurans*, **Lane 10:** *M. gypseum*)

Discussion

The aim of this study was to compare the results achieved from four different freezing protocols, including sterile distilled water, cryopreservation with glycerol, freezing mycobiotic agar, and preserving in the TSB.

Transportation of clinical samples and long-term recoverability of fungal strains are critical to epidemiological studies. In this study, it was assessed a number of methods for preserving culture collections of fungi such as storage in sterile distilled water, cryopreservation with glycerol, preserve in the TSB, and freezing mycobiotic agar. Levels of high viability were obtained with storage in the distilled water and freezing mycobiotic agar. The methods studied in this research for preserving fungal strains have the following advantages: The morphology of the cultures is preserved. Also, the physiological characteristics of all dermatophytes survive had preserved.

The results were different for storage at -20°C and were dependent on the preservative. In other words, choosing a suitable preservative can increase the survival of microorganisms in freezing temperatures.

The results of this study showed the strains have to be matured before storage of fungi. It was found the lowest regrowth rates for *T. schoenleinii, M. ferrugineum, T. violaceum,* and *E. floccosum* which produce only a few or no microconidia. Previous studies have shown the viability of storage dermatophyte cultures depends on the number of microconidia [31]. This data confirm Hasegawa's assumption [32]. Similar difficulties, especially with cultures of *E. floccosum*, *T. schoenleinii,* and *M. ferrugineum*, have been described by Butterfield *et al*. [33] and Gentles *et al*. [34]. Also, the study by Bosmans [35] confirmed the results of this study, since it was found better results when "freezing agar" was used as a storage medium. In addition, Qiangqiang *et al*. [6] showed that the procedure for maintaining dermatophyte cultures in distilled water is better than lyophilization. To achieve success in distilled water preservation, in this experiment, the following conditions are essential. Furthermore, the sterile operation is necessary to prevent contamination. Storage of dermatophyte species shows some peculiarities, such as the transition to pleomorphism or contamination by bacteria or other fungi. However, the contamination with other fungi was seen only in the TSB cultures.

The genetic fidelity was checked by ITS-RFLP. The ITS regions were targeted because mutations are not phenotypically represented in these non-coding segments. All the evaluated specimens were identified at the level of species by the unique banding pattern related to each species .The RFLP patterns were similar to *M. ferrugineum* and *M. canis* because they vary in 2bp in ITS region [30]. The studies by Singh *et al*. [19] and Ryan *et al*. [20] confirms the results of the genetic stability of cryopreserved in the present study.

Conclusion

The procedure to preserve cultures in sterile distilled water is reliable, simple, and inexpensive. The methods including sterile distilled water, cryopreservation with glycerol, freezing mycobiotic agar, and preserving in TSB preserves both the morphology and viability of dermatophyte cultures, also the growth of dermatophytes depends on the methods for culture collections.

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Ethical permissions: The ethics committee of Tarbiat Modares University, Tehran, Iran confirmed the present study.

Conflict of Interests: The author declares that there is no conflict of interests.

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