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**SHORT COMMUNICATION**

# Quantitative preservation of viability of *Aspergillus fumigatus*

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Quantitative preservation of the viability of two isolates of *Aspergillus fumigatus* was studied over a 15-month period of storage. Significant loss of viability occurred in isolates preserved at −70°C in both phosphate-buffered saline with Tween (PBST) and 0.1% gelatin, immediately, and between 6 and 15 months. Storage in 10% and 25% dimethyl sulphoxide at −70°C was successful up to 8 weeks with gradual loss of viability later. PBST at 4°C or room temperature, or 10% glycerol at −70°C maintained 100% viability of the cultures up to 6 months and 15 months, respectively. To preserve 100% viability, long-term storage of *A. fumigatus* is best done in 10% glycerol in PBST at −70°C compared with the other methods tested.

Previous work concerning the viability of fungi over long periods of time has been for the most part initiated by the desire and need to preserve culture collections without loss of rare or particularly valuable isolates. Several approaches have been adopted including submersion of cultures under water, freezing agar cultures at −20°C, lyophilization, storage in liquid nitrogen or drying onto silica gel or sand [10]. Several of these systems have been successful over many years, and in some cases decades.

In our work related to animal models of fungal infection, one of the primary requirements is to give an identical inoculum to different groups of animals on different occasions as parts of the same experiment. In addition, much *in vitro* work depends upon accurate quantitation of inoculum as one aspect of standardization of a particular method. We therefore undertook a prospective study to examine the viability of *Aspergillus fumigatus* conidia in different solutions at different temperatures over periods of up to 15 months.

Two clinical isolates were selected for this work. Isolate 2AF has the DNA type (4,6,A,*Sal*I; 4,6,a,*Xho*I) and 10AF the DNA type (3,9,A,*Sal*I; 5,13,B,*Xho*I) as defined previously [3]. Both isolates had the typical morphology of *A. fumigatus* and both grew at 50°C.

Each isolate was grown on potato dextrose agar (Difco Laboratories, Detroit, Michigan) for a period of 13 days at 35°C, and then for 3 days at 22°C. The conidia were harvested using 5 ml sterile phosphate-buffered saline (pH 7·4) containing Tween 80 (0·2% v:v) (PBST). The culture was flooded with PBST and a sterile loop passed over the surface to disperse the conidia. The PBST-conidia suspension was then aspirated from the plate with a sterile Pasteur pipette and mixed vigorously with a vortex.

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Dilutions in PBST of $10^{-5}$, $10^{-6}$ and $10^{-7}$ were plated onto sheep blood agar (trypticase soy agar with 5% defibrinated sheep red blood cells) (Baxter) and incubated for 48 h at 35°C. Colony counts were performed and the quantitative viability of each isolate was calculated.

The entire volume of the conidial suspension was separated into 2 ml samples and 1 ml of 'preservative' added to each. The 'preservatives' used were 10% glycerol, 0·1% sterile gelatin, 25% dimethyl sulphoxide (DMSO), 10% DMSO (all final concentrations in PBST), or PBST alone. After vortex mixing, each sample with its appropriate 'preservative' was then separated into seven additional samples each of 0·4 ml. These were placed into 2 ml glass screw-top labelled vials. These were stored at −70°C, or in the case of PBST two additional samples stored at +4°C and room temperature, generally 22–24°C.

At selected intervals, a single glass vial from each experimental group was removed from its storage condition and a series of six 10-fold dilutions prepared in PBST. The last three dilutions were plated onto sheep blood agar and inspected at 48 h for growth at 35°C. Colony counts were recorded, viability calculated and compared with the initial viability.

There was an immediate loss in viability of approximately 1-log of both isolates stored at −70°C in PBST or 0·1% gelatin (Fig. 1). In addition, observation of growth after storage showed two colony types; some typical, others very small. Further loss of 1- or 2-logs of viability was seen between 6 and 15 months of storage with these storage conditions.

There was good preservation of viability at −70°C in both 10% and 25% DMSO for up to 8 weeks, with slight loss at 6 months. However, between 6 and 15 months of storage in 10% DMSO resulted in the loss of 1-log of viability and in 25% DMSO the loss of 2-logs of viability (Fig. 1). There was no alteration in colony morphology.

There appears to be only one isolate-related difference in viability, with preservation in 0·1% gelatin at −70°C. Isolate 2AF showed at least a 1-log drop in viability at 6 months and a greater than 3-log fall at 15 months. Isolate 10AF showed about a 1-log loss at 6 months and less than a 2-log fall at 15 months.

Cultures preserved in PBST at 4°C or room temperature were only maintained for 6 months. These conditions preserved good viability (≈ 100%). There was some hyphal growth macroscopically in those cultures preserved at room temperature and uniform colony morphology after 48 h culture on sheep blood agar. Despite macroscopic growth, quantitative viability was preserved for up to 6 months in PBST.

Preservation in 10% glycerol at −70°C yielded excellent preservation of viability of 100% persisting through the 15-month study period as displayed in Fig. 1. There was no alteration in colony morphology. The consistency of the viability determinations was also tested in this experiment and appeared to be highly reproducible.

This study differs from previous studies of the preservation of moulds over time by examining quantitative viability of only two isolates at multiple time points with seven different modes of storage. Buell & Weston [1] extended preliminary work by Sherf [9] by preserving 4500 isolates under mineral oil for up to 2 years with the loss of only one species, *Blakeslea triaspora*. Others have preserved isolates under water with an overall recovery of 93% up to 5 years later [5]. Lyophilization of fungal cultures has been another popular storage method with a 91% recovery after 17–18 years in one study [8]. In this method only conidia and preferably young ones were lyophilized. Freezing either the entire culture [2], or pieces of it, in plasma or serum has also been tried [6].

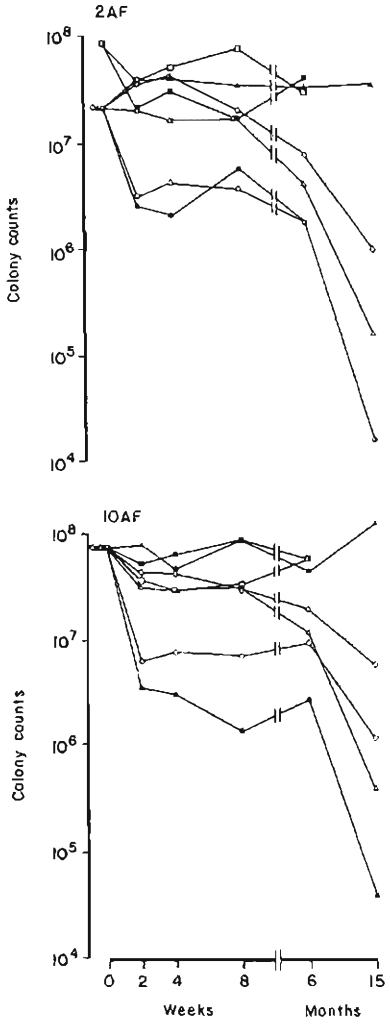


FIG. 1 Quantitative viability (colony counts) over time of two isolates of *A. fumigatus*, 2AF and 10AF, stored in different 'preservatives'. Symbols are the same for both panels. □ PBST at 4°C, ■ PBST at room temperature, ● PBST at −70°C, ○ 0·1% gelatin at −70°C, ◇ 10% DMSO at −70°C, △ 25% DMSO at −70°C and ▲ 10% glycerol at −70°C.

In the former case, 10 (6·1%) isolates of 164 hyphomycetes (thus including *Aspergillus* and *Penicillium)* failed to grow. In 1984, Smith of the Commonwealth Mycological Institute at Kew thoroughly reviewed his experience with a variety of different storage methods including maintenance on agar at 4°C, at −20°C, under oil and in water, maintenance on anhydrous silica gel or in soil/sand, freeze drying, and freezing in liquid nitrogen [10]. *Aspergillus* species appeared to be best preserved under oil and to be unpreservable in liquid nitrogen [10]. Freeze-drying or storage with silica gel were possible alternatives [10]. Recently others have examined the issue. Jones *et al.* [4] preserved blocks of growth on agar under water at room temperature for up to 5 years and 20 of 4018 (0·5%) were non-viable [4]. Medically important fungi including *Aspergillus*

species were stored on potato dextrose agar at −70°C for up to 10 years with the loss of only 33 (2%) isolates [7].

Our study design was adopted to address the question of quantitative viability, not merely the question of growth or no growth. In addition, we were interested in viability over short periods of time. The results indicate that for periods of up to 8 weeks, storage of *A. fumigatus* conidia at room temperature or at 4°C in PBST as well as 10% or 25% DMSO or 10% glycerol at −70°C yielded close to 100% viability. After 6 months, only preservation at room temperature or 4°C in PBST and at −70°C in glycerol were satisfactory. Given the macroscopically visible growth after 6 months at room temperature or 4°C, which probably indicates some alteration in metabolic characteristics over time and/or selection of a subpopulation(s) with altered characteristics from the original inoculum, preservation of 100% viability of *A. fumigatus* for longer periods is best achieved in 10% glycerol at −70°C.

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