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Stability of Hybridization Activity of *Coccidioides immitis* in Live and Heat-Killed Frozen Cultures Tested by AccuProbe *Coccidioides immitis* Culture Identification Test

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Frozen hyphal suspensions of *Coccidioides immitis* were evaluated for suitability as positive control cultures in the AccuProbe *C. immitis* culture identification test. The genetic probe hybridization activity of heat-killed and viable frozen cultures, stored at −20 and −70°C and tested over a 10-month period, was compared to that of a freshly grown culture, and the results were evaluated based upon the manufacturer's established positive and negative photometric light unit (PLU) cutoff values. All *C. immitis* suspensions produced positive hybridization values well above the positive and negative cutoff values, and no significant decrease in hybridization activity was observed with the frozen cultures after 10 months of storage. The frozen, heat-killed suspensions produced PLU values with less variability (coefficient of variation, 8%) over 10 months than the fresh or frozen viable cultures and were deemed the most stable and safe form of positive control material to use.

The laboratory diagnosis of coccidioidomycosis has greatly improved with the introduction of genetic probes for the identification of *Coccidioides immitis* cultures (6). The genetic probe most often used for identification of *C. immitis* is the AccuProbe *C. immitis* culture identification test (Gen-Probe, Inc., San Diego, Calif.). The probe is an acridinium ester-labeled DNA probe which hybridizes to complementary sequences of *C. immitis* rRNA. The test is relatively rapid, sensitive, and specific and requires the use of two highly pathogenic fungi, *C. immitis* and *Blastomyces dermatitidis*, as positive and negative control cultures, respectively. Strict safety measures are required to handle these fungi, including the use of a biological safety cabinet and wetting down of cultures which have produced aerial mycelia to reduce the aerosolization of infectious, dry, airborne arthroconidia (3). Additionally, viable subcultures of these fungi must be maintained by transferring cultures at weekly intervals before aerial growth develops. This study was undertaken to reduce the hazards of manipulating viable cultures and to determine the suitability of frozen hyphal cells for use as controls in the AccuProbe *C. immitis* culture identification test. The two objectives were (i) to determine if both killed and viable frozen hyphal cells could be used as positive and negative controls and (ii) to determine how long frozen killed and live cultures would maintain their hybridization activity at −20 and −70°C over several months of storage (1, 4). Additionally, an attenuated culture of *C. immitis*, provided by Gen-Probe, was evaluated as a positive control organism for the AccuProbe test.

Culture preparation. *C. immitis* ATCC 28868, *B. dermatitidis* ATCC 60916, and an attenuated strain (avirulent in mice) of *C. immitis*, ATCC 96907, obtained from K. Clark, Gen-Probe, Inc., were used in this study. All culture work was performed in a Sterilguard laminar-flow biological safety cabinet. The mold phase of each culture was grown on Sabouraud dextrose agar slants (Remel, Inc., Lenexa, Kans.) for 4 days at 28°C until glabrous mycelium covered the slants. Mycelia from each culture were then transferred to sterile tubes containing 3-mm-diameter sterile glass beads and sterile 20% glycerol. The mycelia were minced into small 1- to 3-mm pieces with a sterile applicator stick and then vortex mixed for 3 min, which resulted in the 1- to 2-mm-size sample suspension recommended by the manufacturer. One aliquot of the mycelial suspension of each strain was killed by exposure to steam heat at 95°C for 15 min in an autoclave under normal atmospheric pressure (2). Aliquots of both heat-killed and viable mycelial suspensions of all three strains were frozen in cryovials and kept for 10 months at −20 and −70°C. The heat-treated suspensions were tested for viability by inoculating portions of each onto Sabouraud dextrose agar slants and incubating them for 1 month at 28°C. All heat-treated cultures were found to be nonviable. Each week, a heat-killed −20°C suspension and a viable −20°C suspension of each fungal strain were thawed and tested for hybridization activity according to the manufacturer's procedure, along with actively growing cultures of *C. immitis* and *B. dermatitidis* maintained on Sabouraud dextrose agar slants at 28°C. At monthly intervals, the −70°C viable and killed suspensions were tested by the same method. All tests were read on a PAL luminometer. Hybridization results were recorded as either positive (greater than 1,500 photometric light units [PLU]) or negative (less than 900 PLU). Quantitative comparisons between weekly PLU readings of *C. immitis* suspensions could not be made because each aliquot of a hyphal suspension containing the target rRNA could not be precisely standardized from one vial to another. Therefore, weekly measurements of hybridization activity were compared to the manufacturer's established positive and negative PLU cutoff values. The weekly values were further evaluated for any significant differences between storage temperatures or change in activity over a 10-month period by using Kruskal-Wallis one-way analysis of variance by ranks and linear regression (5).

Hybridization activity. Results of AccuProbe tests are described in Table 1. The range of positive and negative PLU values represents 27 replicate tests performed two to four times per month for 10 months on aliquots of *C. immitis* ATCC 96907 and *B. dermatitidis* ATCC 60916. Suspensions stored at −70°C were tested nine times, approximately monthly, in a

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TABLE 1. Luminometer values obtained from heat-killed and viable cultures of *C. immitis* and *B. dermatitidis* stored at 28, −20, and −70°C and tested by the AccuProbe *C. immitis* culture identification test[a]

Culture type and maintenance temp (°C)	*C. immitis* ATCC 96907			*B. dermatitidis* ATCC 60916		
	Mean PLU value (range)	No. of determinations	% CV[b]	Mean PLU value (range)	No. of determinations	% CV
Viable						
28	11,546 (4,192–13,934)	27	11	144 (44–264)	27	45
−20	9,263 (3,297–12,318)	27	26	61 (61–155)	22	57
−70	9,547 (6,651–12,069)	9	21	NT[c]		
Heat killed						
−20	10,175 (8,555–11,736)	27	8	56 (22–124)	27	54
−70	9,234 (6,767–11,369)	9	14	NT		

[a] AccuProbe cutoff values: positive, >1,500 PLU; negative, <600 PLU; repeat range, 1,200 to 1,499 PLU.
[b] CV, coefficient of variation.
[c] NT, not tested.

9-month period. All tests performed on both heat killed and viable *C. immitis* ATCC 96907 and ATCC 28868 strains stored at either −20 or −70°C and tested over a 10-month period, produced luminometer readings or PLU values well above the positive cutoff. All tests performed on *B. dermatitidis* stored at 28 and −20°C produced PLU values well below the negative cutoff value. No test results fell into the repeat range, nor was there any consistent decrease in PLU values obtained with *C. immitis* during the 10-month storage period. Using the Kruskal-Wallis one-way analysis of variance by rank for independent paired samples which are not normally distributed, we compared the PLU means of the heat-killed and viable cultures stored at −20°C with the fresh culture at 28°C. This test statistic indicated that the means were significantly different ($P < 0.05$). However, this finding was not surprising given the lack of standardization of rRNA in the three culture suspensions and the relatively large coefficient of variation in test results from week to week. The most important objective was to observe any significant trends in hybridization activity of stored suspensions. This was estimated by linear regression, plotting PLU values (ordinate) as a function of time (abscissa) in weeks. A best-fit line was obtained for each set of data from each of the stored suspensions. For each regression line, a slope (b) was calculated which represented the change in PLU values for each unit of time (in weeks). Heat-killed *C. immitis* suspensions stored at −20°C produced a best-fit line with a positive slope, i.e., $b = 66.7$ PLU/week (0.6% of the mean PLU value), whereas the viable −20°C and fresh 28°C suspensions produced best-fit lines with negative slopes, i.e., $b = -29.9$ PLU/week (0.3% of the mean PLU value) and −74.0 PLU/week (0.6% of the mean PLU value), respectively. These slope values represent relatively small changes in PLU values compared to the magnitude of actual PLU values (Table 1). In light of the large coefficient of variation in test results and the relatively small slope values, we concluded that there was no consistent trend toward decreasing PLU values over the 10-month period with either the heat-killed or viable frozen suspensions which would preclude them from being used as suitable positive controls. In summary, the heat-killed −20 or −70°C *C. immitis* suspensions demonstrated less variability and more stable hybridization activity over 10 months than did the viable suspensions. Additionally, the killed cultures pose less of a biohazard for laboratorians. Longer storage is possible and requires further hybridization studies. This technique of maintaining positive and negative control cultures for genetic probe testing saves preparation time and reduces some of the hazards of frequent subculturing of control cultures. This heat-killing and freezing technique may have further applications in the testing of isolates from patients and the evaluation of additional fungal, mycobacterial, and other microbial probe testing systems.

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