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Separation of *Paracoccidioides brasiliensis* conidia through Percoll gradients

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The conidia of *Paracoccidioides brasiliensis* are the structures most likely to serve as the infectious propagules of this fungus. This study describes our attempts to purify conidia by eliminating mycelial fragments. Purification was attempted using discontinuous 95% and 60% Percoll gradients with densities of 1.167 and 1.107, respectively, prepared either in 0.15 mol/L PBS or 0.25 mol/L sucrose. The best results were observed with the 95% and 90% gradients in sucrose; with the former, conidial purity ranged from 70.6 to 100%, with a mean of 82.3% and a coefficient of variation (VC) of 11.7. With 90% gradients, purity was achieved between 70.4 and 92.5%. The mean in this case was 80.6% and the VC was 9.2%. The use of two consecutive 95% Percoll gradients in sucrose was tested. The recovery efficiency per plate, which averaged 2.5×10^6 conidia per plate with one gradient, increased to $5.1 \pm 1.3 \times 10^6$ conidia with two gradients. The use of Percoll did not affect the viability of the conidia, which was always $\geq 90\%$. This method allows the preparation of a conidial sample almost free from contamination with mycelial fragments, thus facilitating quantitative determination of cause and effect in *in-vivo* interactions between *P. brasiliensis* and its hosts.

Keywords conidia, density, mycelial fragments, *Paracoccidioides brasiliensis*, Percoll gradients, separation

Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a systemic mycosis frequently diagnosed in Latin America. The major endemic areas are located in parts of Brazil, Colombia, Venezuela, Ecuador and Argentina [1]. The disease produced by this dimorphic fungus is usually chronic and progressive and affects several organs and systems. The lungs are the site of the primary infection and the organ most frequently involved [1–3].

It is accepted that the conidia of *P. brasiliensis* are important infectious propagules [4]. It is difficult, however, to isolate conidia from this fungus both in terms of obtaining sufficient quantities for experimental work and in terms of obtaining sufficient purity to be able to attribute results to conidia alone. For this reason, most experimental models of PCM have used yeast cells as inoculum source. However, experimental studies have also been conducted using conidia [4–7] obtained by filtration through glass wool [8]. Such conidial preparations show significant contamination with mycelial fragments (MF). An efficient and reliable method to purify conidial inoculum is required so that precise evaluation of naturalistic experimental models of *P. brasiliensis* infection can be attempted.

Centrifugation through Percoll colloidal silica gradients has been repeatedly used to isolate different kind of cells or other particles [9–14]. This method is based on the differing densities of various types of particles. Physical characteristics related to cell density can be used to purify separate populations of cells in proce-

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dures based on centrifugation through density gradients [9].

The aim of this study was to standardize the purification of *P. brasiliensis* conidia through Percoll gradients so as to obtain conidial inoculum that was as free of contaminating MF as possible.

Materials and methods

Paracoccidioides brasiliensis *culture*

Mycelial cultures of the Colombian *P. brasiliensis* 'Gr' strain (ATCC 60885, American Type Culture Collection, Manassas, VA) were used in this study [8]. The fungus was maintained at 18°C by passage every two weeks on modified Sabouraud's peptone dextrose agar slants (Mycosel Agar, BBL; Becton Dickinson). Fungal growth was amplified by subculturing material from one of these tubes into McVeigh–Morton solid synthetic medium (SMV) [15]. From the mycelial growth obtained on SMV agar, subcultures into liquid SMV medium were then made. These cultures were incubated at 18°C with constant shaking, at 150 r.p.m., for 2 weeks. The mycelial mass obtained was homogenized (Waring Blender, 8400; Eberbach Corporation, Miami, FL, USA), and transferred aseptically to Petri dishes containing media poor in carbohydrate content, such as water agar (Bacto-agar; DIFCO, Detroit, MI, USA) and dextrose salts agar [16]. The cultures were incubated at 18°C for 1–2 months or longer until conidiation had occurred at sufficient levels. The cultures were examined under a stereoscope and plates free of contamination by environmental fungi and bacteria were used to recover conidia.

Collection of conidia

For the harvesting of conidia, dishes with the highest levels of sporulation were chosen. Each harvested dish was flooded with 10 ml 0.15 mol/L NaCl supplemented with 0.01% Tween 20, penicillin (100 U/ml) and streptomycin (100 μg/ml). Fungal material was scraped with a disposable loop and gently removed from the agar surface; this procedure was repeated three times per dish until a final volume of 30 ml had been obtained. The suspension was transferred to a sterile screw-capped Erlenmeyer flask containing glass beads 3 mm in diameter, and was shaken in a reciprocating shaker at 250 r.p.m. for 45 min at 20 ± 2°C. The pulverized suspension was then centrifuged at 1500 *g* at 4°C for 30 min. The pellet was resuspended in 10 ml PBS and sonicated twice at 4°C, 7 Hz for 15 s (Sonicator model 200; Branson Ultrasonic, Danbury, CT, USA) to optimize the detachment of conidia from MF.

Percoll gradient preparation

Different gradients of Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) were prepared. Assays of their effectiveness in separating out conidial inoculum incorporated variables such as the number of culture dishes used to prepare inoculum as well as the type of dilutent used (0.15 mol/L phosphate-buffered saline [PBS] or 0.25 mol/L sucrose). We tested several densities, including 95% (g/ml) Percoll in sucrose (δ [density] $= 1.167 \pm 0.030$), 90% Percoll in 10X PBS ($\delta = 1.144 \pm 0.001$) and 60% Percoll in Dulbeco Minimal Essential Medium (DMEM; Life Technologies, GIBCO BRL, Rockville) ($\delta = 1.107 \pm 0.040$). We also varied the number of gradients used in successive steps, as well as the source of material used in further steps, namely whether use was made of the pellet obtained in centrifugation or of the material collected on the meniscus of the Percoll gradient. The densities of the different Percoll gradients were determined by comparing the weight of 1-ml aliquots of each gradient with the weight of the same volume of distilled water [17].

Paracoccidioides brasiliensis *conidia: purification by discontinuous Percoll gradients*

Initially, 90% and 60% Percoll gradients were assayed in PBS [18]; later, 90% and 95% Percoll gradients in PBS and sucrose were used. In the final assays, attempts were made to use two consecutive 95% Percoll gradients in sucrose for each sample, with the goal of increasing the number of conidia recovered per dish [12,19]. For these assays, the sonicated pellet obtained from each culture dish was diluted in 35 ml PBS and layered over 10 ml of each of the different recently prepared Percoll gradients. The gradients were then centrifuged at 1500 *g* at 4°C for 60 min, and the pellets were washed twice with PBS at 1500 *g* at 4°C for 30 min. In experiments in which a second 95% Percoll in sucrose was used, fungal material from the meniscus of the first gradient was used; this material was removed, diluted in PBS and pipetted over a new gradient consisting of 95% Percoll in 0.25 mol/L sucrose. After washing of pellets, conidia and MF were counted in a haemocytometer. The purity of the conidia and the recovery efficiency were calculated. The latter is defined as the absolute number of conidia obtained per starting culture dish. Viability of the conidia was evaluated by staining with fluorescein diacetate and ethidium bromide [20].

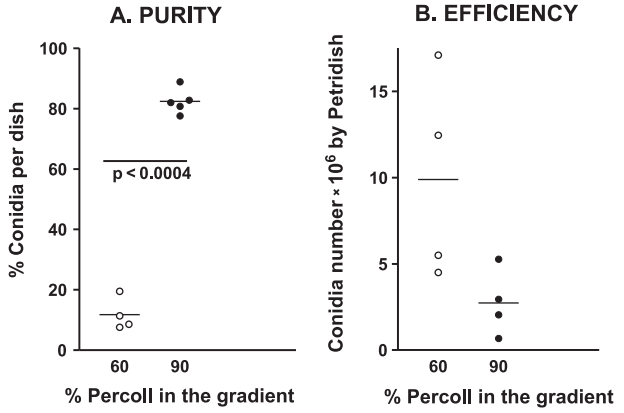

Fig. 1 *Paracoccidioides brasiliensis* conidia recovered by Percoll discontinuous gradients: 90% Percoll in 0.15 mol/L PBS and 60% in Dulbeco Minimal Essential Medium (DMEM). Values obtained for purity (A), defined as the percentage of conidia free of MF recovered, and efficiency (B), defined as the number (in millions) of conidia recovered per starting culture dish, indicate that maximal purity but relatively low efficiency was obtained via use of the 90% Percoll gradient; $n = 4$ experiments per treatment, and $P < 0.0004$ for the significance of the difference in results. Solid dots: *P. brasiliensis* conidia recovered using a 90% Percoll gradient in 0.15 mol/L PBS. Open dots: *P. brasiliensis* conidia recovered using a 60% Percoll gradient in DMEM.

Statistical analysis

For the various experiments, two parameters were evaluated: firstly, purity, defined as the percentage of conidia free of MF recovered, and secondly, efficiency, defined as the number (in millions) of conidia recovered per starting culture dish. A one- or two-way analysis of variance (ANOVA) was done. For all statistical analyses Prism 3 software (GraphPad Software, San Diego, CA, USA; 1999 version) was utilized.

Results

Initial experiments

The initial gradients used were discontinuous gradients of 90% Percoll in 0.15 mol/L PBS and 60% Percoll in DMEM. The 90% Percoll gradient allowed a high rate of conidial recovery (83.6%), significantly higher ($P < 0.0004$) than the rate obtained with the 60% gradient (12.5%) (Fig. 1). In terms of efficiency, lower conidial numbers (2.7×10^6) were obtained with the 90% gradient than with the 60% gradient, which yielded 9.9×10^6 conidia per starting Petri dish. The highest concentration of viable conidia showing the lowest level of MF contamination was obtained with the 90% gradient. This result indicated that conidial material had a density greater than or equal to the density of the 90% gradient, which was 1.144 ± 0.001.

Effect of the dilutent (PBS or sucrose) on the recovery of P. brasiliensis *conidia through Percoll gradients*

Based on the previous results, Percoll gradients at 90% and 95% were prepared in either 0.15 mol/L PBS or 0.25 mol/L sucrose. We wanted to compare the effect of different diluents since Percoll manufacturers suggest that certain cells tend to aggregate in the presence of salts. As shown in Fig. 2, the 95% Percoll gradient in 0.25 mol/l sucrose yielded conidia with a purity varying between 70.6 and 100%. The mean purity was 82.3% and the coefficient of variation (VC) was 11.7%. With the 90% Percoll gradient in 0.25 mol/L sucrose, the purity ranged from 69 to 72.4%, with a mean of 70.4% mean and a VC of 9.2%. For gradients prepared in PBS with 95% Percoll purities varied from 69% to 72.4%, with a mean of 70.4%. With 90% Percoll gradients in PBS, the purity ranged from 73 to 75.5%, with a mean of 74.1%. Overall, the 95% Percoll gradients in sucrose produced conidial suspensions that had statistically significantly higher ($P < 0.05$) purity levels than were obtained with the corresponding PBS gradient. In terms of efficiency, both the gradients prepared in sucrose increased the recovery of conidia as compared to the equivalent gradients prepared in PBS.

Increase in conidial recovery via use of two consecutive 95% Percoll gradients in sucrose

With the goal of increasing the recovery of conidia, a second Percoll gradient was done using material that, as previously indicated, remained on the meniscus of the first 95% Percoll gradient in sucrose. The efficiency

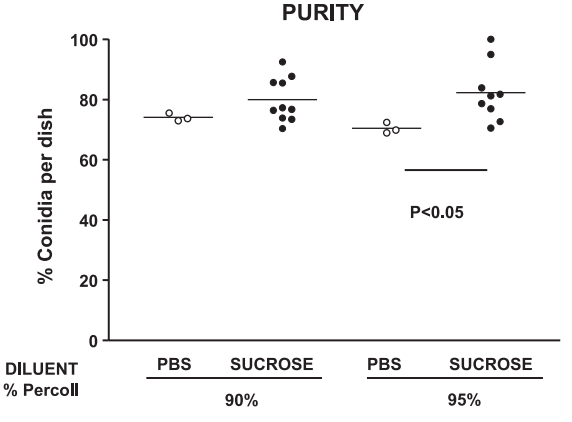

Fig. 2 Effect of the dilutent on the purity of *Paracoccidioides brasiliensis* conidia in Percoll gradients. Data show that a 0.25 mol/L sucrose diluent in 95% Percoll ($n = 9$ trials) but not in 90% Percoll ($n = 3$) is superior to 0.15 mol/L PBS ($P < 0.05$) in the separation of conidia. Solid dots: *P. brasiliensis* conidia obtained using a Percoll gradient in 0.25 mol/L sucrose. Open dots: *P. brasiliensis* conidia obtained using a Percoll gradient in 0.15 mol/L PBS.

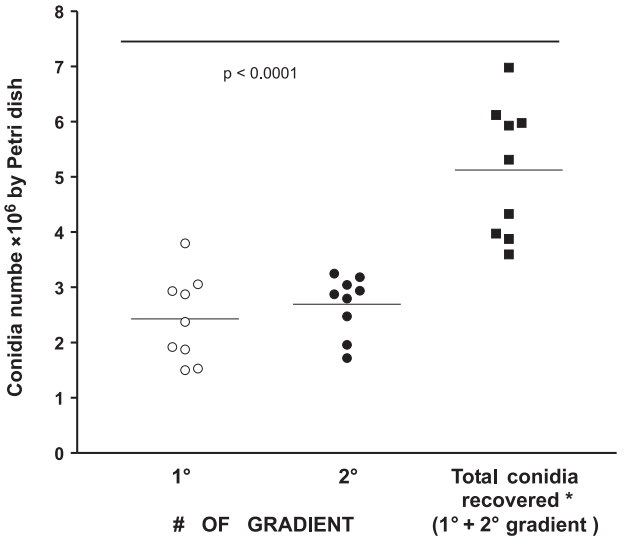

Fig. 3 Number of *Paracoccidioides brasiliensis* conidia recovered through use of two consecutive 95% Percoll gradients in 0.25 mol/L sucrose. A significantly higher ($P < 0.0001$) number of conidia were obtained when two consecutive Percoll gradients, the prepared from material obtained from the meniscus of the first after centrifugation, as outlined in the text, were used ($n = 9$ trials). Open dots: *P. brasiliensis* conidia obtained with the first 95% Percoll gradient in 0.25 mol/L sucrose. Solid dots: *P. brasiliensis* conidia obtained with the second 95% Percoll gradient in 0.25 mol/L sucrose. Solid squares: *P. brasiliensis* conidia obtained with two consecutive 95% Percoll gradients in 0.25 mol/L sucrose.

of recovery of conidia from the first and second gradients, considered separately, was similar: 2.3×10^6 and 2.6×10^6 per culture dish, respectively (Fig. 3). The total conidia number obtained per culture dish thus increased ($P < 0.0001$) with the use of two consecutive 95% Percoll gradients in sucrose ($5.12 \pm 1.32 \times 10^6$ conidia) (Fig. 3). In the second gradient the level of purity was conserved and the viability was always $\geq$ 90%.

Discussion

The method originally recommended to obtain *P. brasiliensis* conidia was based on filtration through glass wool [8]; however, this method yields conidial preparations with relative high quantities of contaminating MF. Such inocula may not be appropriate for certain experimental purposes. Compared to the glass wool method, the methods described here make it possible to obtain suspensions that have higher conidial numbers and less contamination with MF.

In general, one basic factor in separation and purification techniques is to determine the relative density of the cells or subcellular particles that are to be separated. Separation techniques based on centrifugation through Percoll gradients are useful to segregate cells or subcellular particles that are of unknown density [17]. Initial experiments are generally done to define the density intervals where the materials of interest may be found; in the present study, comparison of results obtained with 90% and 60% Percoll gradients indicated that the density of *P. brasiliensis* conidia was $\geq 1.144 \pm 0.001$. It was then decided to assay 90% and 95% Percoll gradients in 0.15 mol/L PBS and 0.25 mol/L sucrose. Manufacturer's instructions for Percoll indicate that the separation of certain cells is achieved more efficiently through sucrose. This proved to be applicable in our case, in that the best recovery rate we obtained was with a 95% Percoll gradient in 0.25 mol/L sucrose (density = 1.167). Additionally, the use of two consecutive 95% Percoll gradients in 0.25 mol/L sucrose increased the efficiency of the procedure. The viability of the conidia in all experiments was $\geq 90\%$, indicating that Percoll did no damage to conidial integrity.

The use of 95% Percoll gradients in sucrose allows recovery of *P. brasiliensis* conidia with minimal MF contamination. This method is recommended for experiments aimed at determining the role of conidia in host-*P. brasiliensis* interactions.

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