RESEARCH NOTE

IN VIVO CULTURE OF *CRYPTOSPORIDIUM* OOCYSTS FOR LABORATORY USE

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Abstract. In the present study, we describe *in vivo* cultivation to produce oocysts. Seven-day-old mice were orally infected with 100,000-120,000 *Cryptosporidium* oocysts. On day8 post-infection, the mice were killed by ether, and the small and large intestines collected. A simple extraction procedure was used and purified using Ficoll gradient centrifugation. After purification, the oocysts were preserved in phosphate buffered saline with antibiotic at 4˚C before use.

INTRODUCTION

Cryptosporidium species are protozoa that cause diarrhea in humans, domestic and companion animals worldwide. A major species infecting humans and many mammals is *Cryptosporidium parvum*. The infective stage of this parasite is the oocyst, and the agent reproduces in the gastrointestinal tract. It is excreted with feces, allowing the organism to persist in the environment. Transmission of disease is via fecal-oral route, by drinking or eating food contaminated with infective oocysts. The symptoms are most severe in immunocompromized individuals, especially people infected with HIV.

Studies of *Cryptosporidium* are now becoming of interest to researchers, such as their life cycle, *in vitro* cultivation, elimination methods, drug treatment interval and molecular techniques. It is essential to produce and maintain large amounts of oocysts. Animal models are considered the gold standard for detecting the viability and infectivity of *Cryptosporidium*, especially neonatal animals. To obtain large oocyst yields, the majority of isolations are derived from experimentally infected animals. In this study, we report a simple *in vivo* method to provide a good yield of oocysts for laboratory use.

MATERIALS AND METHODS

The first batch of *Cryptosporidium* oocysts was kindly provided by the Division of Veterinary and Biomedical Sciences, Murdoch University, Australia.

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Infection of mice and collection of oocysts

This was conducted according to Meloni and Thompson (1996). IRC, 7-day-old mice were inoculated with 100,000-120,000 oocysts using a small pipette. On day8 post-infection, the mice were killed by ether exposure and the small and large intestines removed into a Petri dish. The samples were dissected into smaller segments and pooled into a 200 ml beaker and PBS was added (approximately 4 ml/mice). Sputasol (0.005 g/ml of suspension) was added, then the suspension was thoroughly homogenized. The homogenate was poured into a centrifuge tube, left at room temperature for 90-120 minutes on a rotary mixer, then centrifuged at 3,000 rpm for 10 minutes. The supernatant was removed into a separate tube. Ether was added to the tube and shaken vigorously for at least 1 minute. After that, the tube was centrifuged at 3,000 rpm for 10 minutes, shaken again for at least 30 seconds, and centrifuged at 3,000 rpm for 10 minutes. The supernatant layer was removed and the pellet resuspended in 10 ml PBS and transferred to a new tube. The oocysts were stored at 4 ˚C until further purification.

Purification of oocysts

Oocysts were purified using Ficoll gradients, which were formed by layering 1 ml of 4.0, 2.0, 1.0, and 0.5%, respectively. The gradient was spun at 35,000 rpm at 4 ˚C for 5 minutes to help the layer separate. Two ml of oocyst suspension was layered onto the top of the Ficoll gradients and centrifuged at 2,500 rpm at 4 ˚C for 40 minutes. The oocysts were collected from the 0.5% layer and removed into a clean tube. PBS was added and the tube was centrifuged at 3,000 rpm for 10 minutes. The supernatant was removed and resuspended with cold sterile PBS/antibiotic and counted. The oocysts were stored at 4˚C until used.

RESULTS

After the purification method, we used a standard

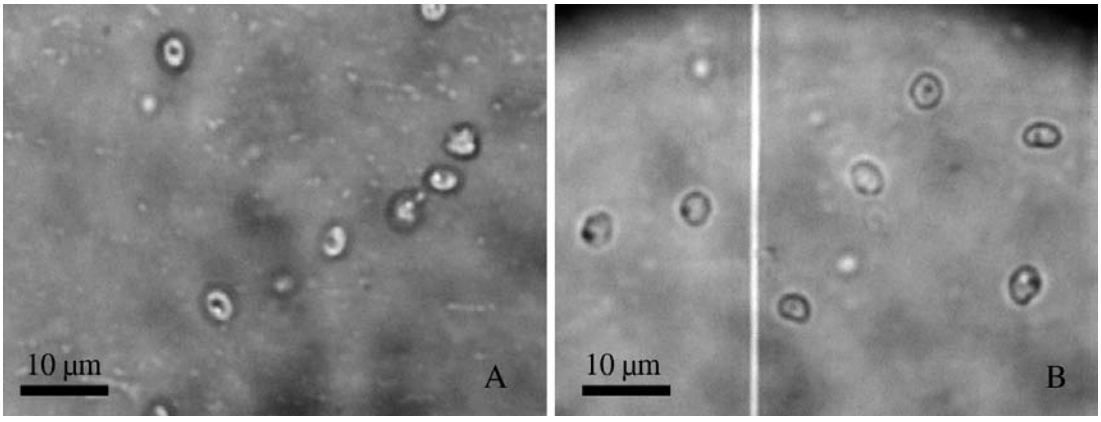

Fig 1- (A,B) Appearance of *Cryptosporidium* oocysts in phosphate buffer saline after purification by Ficoll gradient.

light microscope to observe and count the oocysts using a hemocytometer under ×400 magnification. The oocyst yields isolated from the neonatal mice were about 173,333 oocysts/ml. The oocysts measure 4-6 μm and were round in shape (Fig 1).

DISCUSSION

In the present study, we described an IRC mice model where the animals were inoculated with *Cryptosporidium* oocysts and the oocysts were isolated later. Neonatal animals are susceptible hosts for *Cryptosporidium* (Sherwood *et al,* 1982) and they can generate a large number of oocysts. In the past, experimentally infected neonatal calves or lambs have been used to produce oocysts, and they provided a high oocyst yield. However, large neonatal animals can be relatively expensive, require appropriate animal housing facilities, and skilled personnel to approach them. Suckling mice have been used in many experimental studies of *Cryptosporidium*. Oocysts can be generated and isolated from their guts (Meloni and Thompson, 1996). Adult mice with congenital immunosuppressive mutations, such as nude or scid mice, and mice with steroid-induced immunosuppression, are susceptible to infection. The oocyst yields were detected and collected from their feces (Petry *et al,* 1995).

On the other hand, cell-culture technology has developed into a tool that can be used to study the organism in an environment most similar to the *in vivo* situation. It has successfully developed all stages of *C. parvum* (Hijjawi *et al,* 2001, 2004), *C. hominis* (Hijjawi *et al,* 2001) and *C. andersoni* (Hijjawi *et al,* 2002). *In vitro* cultivation can infect and maintain several species of *Cryptosporidium*, while *in vivo* methods require a specific host for infection. However, it was characteristic of the oocyst when using the cell culture method that mostly thin-walled oocysts were produced, with low numbers of thick-walled oocysts. So, *in vivo* cultivation is a method worthy of consideration for generating and isolating oocyst yields for laboratory use (Meloni and Thompson, 1996; Sursesh and Rehg, 1996).

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