

# CYCLOSPORA CAYETANENSIS: OOCYST CHARACTERISTICS AND EXCYSTATION

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**Abstract.** In Thailand in 1999-2000, *Cyclospora* oocysts from two HIV-infected patients and one patient with prolonged diarrhea were detected by formalin-ether concentration technique. Sporulation was performed by mixing stool samples in 2.5% potassium dichromate solution, sporulated oocysts were then treated with various solutions before mechanical rupturing in order to establish excystation, fewer than 10% of the sporulated oocysts could be excysted. Our techniques provided more details of the characteristic appearance of sporocysts and sporozoites within the oocysts (DMSO- modified acid-fast technique with our modification).

## INTRODUCTION

*Cyclospora*, a coccidian protozoan of humans, is known to cause diarrhea. Cyclosporidiasis has been reported from many areas of the world, although relatively few *Cyclospora* cases were reported in Thailand during the period 1994-1996 (Berlin *et al*, 1994; Wanachiwanawin *et al*, 1995; Morakote *et al*, 1995; Manatsathit *et al*, 1996). Surprisingly, there has been no report of *Cyclospora* detection since 1996. The diagnosis of *Cyclospora* infection relies on detecting the organism in stool specimens and, therefore, it is essential that fecal specimens are collected and handled properly to ensure that *Cyclospora* can first be found and then identified by wet preparation and staining. Microscopic detection of oocysts in the stool is the standard procedure for diagnosis (Eberhard *et al*, 1997). Wet preparation and staining are used to show the *Cyclospora* oocysts (Long *et al*, 1991; Berlin *et al*, 1994; Ortega *et al*, 1997). Staining by the modified acid-fast technique used for *Cryptosporidium* has also been recommended for *Cyclospora*, although the oocysts may be collapsed or distorted and their walls may be less evident than when seen in a wet preparation (Eberhard *et al*, 1997). The purpose of our study is the sporulation and excystation of *Cyclospora* oocysts which enhance the staining of *Cyclospora* sporulated oocysts. The sporocysts and sporozoites could be stained clearly after processing in excysting solutions. This character of sporulated oocysts have never been described before.

## MATERIALS AND METHODS

### Collection of oocysts

One hundred stool samples were collected during 1999-2000. *Cyclospora* oocysts were found from a patient with prolonged diarrhea from the Hospital for Tropical Medicine, Bangkok and two HIV-infected patients from a private hospital. Unsporulated oocysts were detected by direct wet mount and the formalin-ether concentration technique. DMSO-modified acid-fast stain was performed in order to study the oocysts (Bronsdon, 1984); the stool specimens were then mixed in 2.5% potassium dichromate and kept at room temperature prior to the confirmation of sporulation and further excystation.

### Purification of oocysts

The sporulated oocysts were separated from fecal debris using 1 M sucrose as follows: three milliliters of sucrose solution were pipetted into a centrifuge tube and two milliliters of 2.5% potassium dichromate containing sporulated oocysts were layered on the top of sucrose solution. The tube was centrifuged at 800g for 5 minutes and the oocysts that concentrated at the emulsion-sucrose interface were removed and transferred to a clean tube. To each tube, distilled water (1:10) was added and the oocysts were sedimented by centrifugation at 800g for 10 minutes. The oocysts were then washed twice in distilled water. After the supernatant was discarded, the pellet was resuspended in a sterile tube containing 3% sodium hypochlorite to kill other microorganisms. Tubes containing *Cyclospora* oocysts in 3% sodium hypochlorite solution were chilled in an ice bath for 10 minutes and then washed three times with a phosphate buffer saline that was free of calcium and magnesium. The process of purification was performed using an aseptic technique.

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### Excystation

The pellet of oocysts was suspended in synthetic gastric juice containing 0.4% pepsin in hydrochloric acid (pH 1.65), and kept at 37°C for 1 hour; this suspension was made by a sterile technique. The oocysts were removed from the synthetic gastric juice, washed three times using the phosphate buffer saline as previously described and centrifuged at 800g for 10 minutes. The sediment of oocysts was resuspended in an excysting fluid that contained 0.5% trypsin and 1.5% sodium taurocholate in MEM medium without serum (Ortega *et al*, 1993). The treated oocysts were incubated at 37°C in a CO<sub>2</sub> incubator for 2 hours to allow the sporozoites to emerge. The sporozoites that were liberated from sporocysts by mechanical rupturing were inoculated into a MDCK cell line and incubated at 37°C in a CO<sub>2</sub> incubator.

### Staining

Some sporulated oocysts were harvested from the excysting fluid and washed three times with phosphate buffer saline. The sediment containing the sporulated oocysts was smeared onto a clean slide and dried at 85°C overnight before staining by a DMSO-modified acid-fast technique. Several oocysts were ground with a Teflon-coated tissue grinder in order to break them up. The sporocysts and sporozoites that were released from the ground sporocysts were stained using a DMSO-modified acid-fast technique as previously described.

### RESULTS

The macroscopic examination of fecal specimens was made. It was found that the stool that contained *Cyclospora* oocysts was less mucous compared to stool that contained *Isospora*, *Cryptosporidium*, and *Microsporidia*.

Wet mount examination revealed spherical organisms (8-10µ in diameter). Cysts were recognized as unsporulated oocysts. DMSO-modified acid-fast staining was atypical except for the size of the cysts.

Less than 50% of the *Cyclospora* oocysts could be developed as sporulated oocysts after having been kept in 2.5% potassium dichromate at room temperature; excystation was not successful.

After sporulation, light microscopy revealed that some oocysts had two sporocysts with globules inside. Staining of the *Cyclospora* oocysts by a DMSO-modified acid-fast stain revealed the same phenomenon, even when the sporulated oocysts were heated as recommended by Eberhard *et al* (1997). The sporulated oocysts showed atypical phenomenon before they were processed in various solutions. After the sporulated oocysts were treated in sodium hypochloride, synthetic gastric juice and excysting fluid, the oocysts clearly revealed the morphological characteristics of sporocysts with sporozoites [DMSO-modified acid-fast staining with our modification (Fig 1)]. Unsporulated oocysts that were treated to the same procedure also showed more distinct globules.



Fig 1- *Cyclospora cayetanensis*; sporulated oocyst, DMSO-modified acid-fast staining with modification.

Sporocysts and sporozoites were liberated from sporulated oocysts by mechanical rupturing: the rate of excystation by this method was less than 10%. Some sporocysts were still intact and stained pink with a knob at one end of each sporozoite. We found no evidence to suggest that the MDCK cells had been infected by *C. cayetanensis*.

## DISCUSSION

Techniques used for the concentration of *Cyclospora* oocysts, such as formalin-ether and sucrose gradient centrifugation, have been reported by many researchers (Eberhard *et al*, 1997). Our use of both of these techniques was dictated by the needs of our study. The formalin-ether method was selected when the oocysts were identified by wet mount and staining; sucrose centrifugation was appropriate for the study of sporulation and excystation. Formerly, there was no staining technique that could demonstrate sporozoites inside sporocysts, due to the thick wall of the oocyst, which does not allow the dye to penetrate. The cyst wall was not as delicate as it was demonstrated. Some researchers have reported that the oocyst wall is very resistant and that the internal wall is semi-permeable and protects the zygote or sporozoites against the effects of various chemical substances, while the external wall protects it mainly against mechanical damage. Most chemical substances penetrate the external wall (Kheysin, 1972) and for this reason the oocyst was better stained after processing in the various solutions. The process of excystation in our study permitted the staining chemicals to pass through the wall of the oocyst because, after processing, the outer part of the oocyst became thinner and more transparent. In addition, the procedure of keeping treated oocysts at 85°C overnight enhanced the dye's penetration into the cyst wall.

On the basis of our observation, there were many possibilities for bringing about excystation. One is the association with bacteria in fecal specimens during sporulation. Sporulated oocysts should be separated from fecal debris after 10 days using sucrose centrifugation as described by Eberhard *et al* (1997) in order to prevent the degeneration of oocysts; these oocysts should be kept at 4°C after separation. Excystation was attempted using excysting fluid following the Ortega method (Ortega *et al*, 1993). Unfortunately, our attempts were unsuccessful; we therefore incubated the oocysts in another solution that used for the excystation of *Giardia* cysts (Siripanth *et al*, 1995), before we applied the excysting fluid which was followed by mechanical rupturing. These steps gave results in successful excystation.

The failure of excystation may have been due to the viability of the oocysts. The factors which affected the viability can be considered into two groups. The first group of factors is related to the property of oocyst itself. All three cases had a low percentage of oocysts in the stool and only less than 50% of the unsporulated oocysts could be developed into sporulated oocysts. This suggests that there might be some factors that influence the survival of sporozoites, such as the stool not being freshly collected or inappropriately handled, as described by Eberhard *et al* (1997). The second group of reasons is related to the damaging of sporozoites during excystation. These reasons should be further studied. In 2000, Eberhard *et al* attempted to establish experimental *C. cayetanensis* infection in various laboratory animals (*eg* mice, rats, chickens, ducks, rabbits and monkeys) and they concluded that none of these animals was susceptible to infection with *C. cayetanensis*. Therefore the failure of cultivation was due to MDCK which is dog's kidney cells and is not susceptible to *C. cayetanensis* which is recognized to be strictly host specific.

The concentration method for the recovery of parasitic ova and protozoal cysts has improved the ability to detect *Cyclospora* oocysts. Wet preparation and examination by high-power microscopy is recommended in suspected cases. Sporulation should be performed and this should be followed by wet preparation in order to arrive at a final diagnosis. Although the detection of *Cyclospora* oocysts in wet preparation can be greatly enhanced by using epifluorescence microscopy, but most of smaller laboratories including in Thailand can not afford to have this necessary equipment. The reason for the limitation of cyclosporidiasis case reports in Thailand may due to the lack of experience and awareness of laboratory workers. Our findings will hopefully stimulate microscopists to consider the possibility of *Cyclospora* infection in Thailand, especially in HIV-infected patients and patients with prolonged diarrhea.

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