

Cloning of Thai Strain *Giardia intestinalis*

Chutatip Siripanth, Thaiyooth Chintana, Yodsawee Tharaphan and Amorn Lekkra

Various techniques have been developed over the past decade for *in vitro* cultivation of *Giardia intestinalis*.¹ The important factors contributing to the success of such *in vitro* culture include supplementation of the culture medium with various essential nutrients such as bile salts, vitamins and serum and the control of bacterial and fungal contamination. The supply of the above-mentioned essential nutrients to the culture has changed the requirement for co-existing yeast in monoxenic culture of the parasite to axenic culture.² In 1976, Robert-Thompson *et al.*³ introduced the *in vitro* technique to induce excystation of *G.intestinalis* cysts to trophozoites, while Keister⁴ used bile supplementation in the axenic culture of the trophozoites. This information enables isolation, amplification and maintenance of a large number of *G.intestinalis* isolates. However, studies of parasite attributes, eg genes, isoenzymes, mutagenesis, drug sensitivity, pathogenicity, host-parasite relationship, protective vs diagnostic antigens and others, require established parasite clones. In 1980, Gillin and Diamond⁵ described the technique

SUMMARY : Axenic cultures of *Giardia intestinalis* trophozoites were successfully established from human fecal specimens and rectal swabs from dogs using sucrose gradient centrifugation to separate the cysts from fecal material, the excystation method of Robert-Thompson *et al.*³ and culture of the preparation in TYI-S-33 medium supplemented with human serum, vitamin mixture and piperacillin and amikacin antibiotics, respectively. Fungal contamination could be controlled by amphotericin B at 10 µg per ml of medium. Clones of the parasites were obtained using a combination of dilution method and micromanipulation technique.

for monocloning of *G.intestinalis* using a semisolid agar and found that the colony forming efficiency was 20-40%. Later in 1988, Baum *et al.*⁶ introduced the dilution technique for *G.intestinalis* cloning. In this communication, the technique of Baum *et al.*⁶ was adopted with modifications for cloning of a Thai isolate of *G.intestinalis*.

SPECIMENS AND METHODS

Cysts of *G.intestinalis* were collected from freshly passed stools of infected individuals and rectal swabs from dogs. Only stool specimens which contained more than 10 cysts per one 450 × microscopic field were further used for cloning. Each selected specimen was emulsified in 0.85% saline solution, filtered through 16 layers of gauze in

order to eliminate large particles. Ten volumes of distilled water were added to the filtrate and the preparation was centrifuged at 400 × g at room temperature for 10 minutes. The sediment which contained the cysts was rewashed as above for two more times. The cysts in the last sediment were separated from other fecal material by the sucrose-gradient centrifugation technique described by Robert-Thompson

From the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

Correspondence : Chutatip Siripanth, Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

*et al.*³. The cysts were collected and resuspended in the excystation fluid (0.4% pepsin in HCl, pH 1.65) and kept at 37°C for 1–2 hours. The preparation was centrifuged at 200×g for 10 minutes and the cysts were washed two times with distilled water by centrifugation, then they were layered in an 11 × 100 mm glass tube containing 6 ml of TYI-S-33 medium⁴ which was freshly prepared aseptically by adding 10 ml of millipore filtered, heat-inactivated human serum (56°C, 30 minutes), 2.5 ml of NCTC-135 vitamin mixture, 1.0 mg of piperacillin and 1.0 mg of amikacin to 100 ml of sterile broth. The tube was plugged with rubber stopper and incubated at 37°C at 15 degree inclining position from the horizontal plane. The medium was changed daily during the first three days and care was taken not to disturb the sediment. The culture was examined daily under 100 × inverted microscope. Subcultures were made when the pH of the culture fluid dropped to less than 6.4 as indicated by the change of the medium colour (0.2% phenol red was used as an indicator).

Fungal contamination was sometimes found in the cultures. When this occurred, addition of amphotericin B was needed. Subcultures were made according to the number of trophozoites and the degree of fungal contamination. If the original culture contained a large number of trophozoites, subcultures were made by transferring one third of the culture from the original tube into each of two other test tubes. The 6 ml of fresh medium were added to the original tube and to 2 subculture tubes. If the culture contained a moderate number of trophozoites, subcultures were made by transferring half of the culture to another test tube, and then fresh medium was added to both tubes. If the culture was overgrown by fungi, subcultures were made by discarding 5–6 ml of culture fluid, and then

the fresh medium plus amphotericin B was added to give a final concentration of 10 µg per ml. If there was a large number of trophozoites in culture tube which had an overgrowth of fungi, half of the culture fluid was transferred to another test tube and medium plus amphotericin B was added to each tube to give a concentration of 5 µg per ml. After the culture was maintained monoxenically (trophozoites + fungi) for 10 days, *G.intestinalis* trophozoites attached themselves tightly to the glass. At this time, axenic culture could be obtained by discarding all of the cultured fluid which contained suspended fungi from the tube. The tube was then extensively washed with culture fluid containing reducing amounts of amphotericin B from 10, 5, 2 to 1 µg per ml by centrifugation at 200×g for 10 minutes several times. The axenic culture was established once it was found that there was no fungal growth after plating the culture medium onto the Sabouraud's medium. Bacterial contamination was also checked by plating the culture medium onto McConkey and blood agar or inoculation of small aliquots into thioglycolate broth.

After establishing the axenic culture, each culture was serially diluted with fresh culture medium containing antibiotics (piperacillin and amikacin) into sterile test tubes. Small amount of the diluted culture was dropped onto the inside of the lid of the plastic tissue culture plate using a narrow, hook-shaped tip pasteur pipette. Individual *G.intestinalis* trophozoites were sucked up into the pipette under an inverted microscope (200×) using micro-manipulation technique. Each trophozoite was placed into a small sterile test tube containing fresh culture medium with antibiotics. Each tube was immediately plugged with rubber stopper (as it was found out that excessive exposure to oxy-

gen affected multiplication of the trophozoites) and incubated at 37°C in the 15 degree inclining position. The culture was examined daily without changing the medium during the first three days. The medium was changed on the fourth day without disturbing the multiplying trophozoites at the bottom of the tube.

RESULTS AND DISCUSSION

From a total of 200 human fecal specimens examined, only 30 specimens contained more than 10 cysts per 450 × microscopic field. From these, only 7 isolates of *G.intestinalis* were successfully established as axenic cultures and subsequently cloned.

Cysts in rectal swabs from dogs were treated similarly to human specimens for excystation and establishment of axenic cultures. All isolates and clones from both human and dog specimens were kept in liquid nitrogen tank for further studies.

From experience, it was observed that excess amphotericin B (concentration 25 µg/ml of culture medium) caused damage to the *G.intestinalis* trophozoites. Successful control of fungal contamination was obtained when the fungicidal agent was used at 10 µg per ml of the medium. The bacteria encountered in the monoxenic (or dixenic) cultures were gram negative of which the most dominant one was *Pseudomonas* spp. The use of piperacillin and amikacin combination gave satisfactory outcome in the elimination of the bacterial contamination. Phosphate buffered saline containing antibiotics but free from calcium and magnesium ions could be used instead of the culture medium to wash off the fungal contaminants from the culture tubes when the trophozoites have all adhered to the glass surface. After elimination of the contamination, the axenic culture reached its peak within few weeks, when subculture was required.

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