

AXENIC CULTURE AND CRYOPRESERVATION OF *GIARDIA LAMBLIA* ISOLATED IN IRAN

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ABSTRACT

Giardia lamblia cysts were isolated from fecal samples of 55 symptomatic and asymptomatic patients with giardiasis. The cysts were harvested and purified using the sucrose gradient method.¹⁵ The excystation of *G. lamblia* was obtained by treating them with HCl (0.01N)¹, then they were transferred on to modified TYI-S-33 medium. Out of 55 stool samples 17 cases were excysted with a rate of 4-32%, but we were able to cultivate only one case with 12% excystation in culture medium. The generation time of *G. lamblia* was calculated to be about 10.33 hours.

Cryopreservation of trophozoites was successfully done in liquid nitrogen and the thawing process had a survival rate of 85-90%.

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INTRODUCTION

Giardia lamblia is the first reported intestinal protozoan with a world-wide distribution.^{3,4,20} *Giardia* infection may be completely symptom-free or associated with serious diarrhea leading to malabsorption. It produces more clinical manifestations in children than in adults. Since parasitological tests cannot detect more than 50%-75% of cases,^{2,10} researchers continue seeking a more accurate immunological test for its diagnosis. The basis of these techniques depends on availability of pure antigens in sufficient quantities. Axenic culture of *giardia* is the key to the production of these antigens.

Karapetian was the first to report successful axenic cultivation of *G. lamblia*, symbiotically with chick fibroblasts and *Candida guilliermondii*.^{7,13} Subsequently, in 1976 axenic culture of *giardia* by E.A. Meyer made it possible to investigate the different aspects of this parasite.¹⁴ In the present study our goal was to cultivate and maintain an Iranian strain of *G. lamblia* in the laboratory in order to produce pure antigens for use in immunodiagnostic assays and other research purposes.

MATERIALS AND METHOD

Purification *Giardia lamblia* cysts were isolated from the feces of patients with high cyst excretion.⁶ Stools were broken up in tap water and were filtered through two or three layers of cheese cloth gauze. 3 ml of the fecal suspension was layered on 3ml of 0.85 M sucrose¹⁷ and centrifuged at 600 g for 10 min at room temperature (preferably 4°C)¹⁸, then the cysts were collected from the layer between the sucrose-water phase. Washed cysts were carefully added on the top of a discontinuous density gradient consisting of two 3ml layers of 0.85 M and 0.4 M sucrose. After centrifugation at 600 g for 10 min, cysts concentrated at the 0.85-0.4M sucrose interface were collected and washed again. Purified cysts were resuspended in distilled water (D.W.) and stored at 4°C.⁶

Excystation and Cultivation In this study we used the excystation procedure of Bingham and Meyer.^{1,3} Concentrated cysts were diluted 1:10 with HCl (0.01N). The preparation was incubated at 37°C for one hour and centrifuged at 600 g for 10 min. The supernatant was removed and

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the pellet washed with D.W. and about 10^4 - 10^6 cysts were inoculated into the 13×100 mm screw-capped borosilicate tube containing TYI-S-33 culture medium. The modified version of TYI-S-33 medium was used for the culture of *G. lamblia*.⁹ Each 100 ml of medium contained the following: 100mg K_2HPO_4 , 60mg KH_2PO_4 , 2 gr trypticase, 1.0 g yeast extract, 1.0 gr glucose, 200 mg NaCl, 200 mg cystein-HCl monohydrate, 20mg ascorbic acid, 2.28mg ferric ammonium citrate, 50-100 mg dehydrated bovine bile and 10 ml of inactivated bovine serum (pH: 7.0-7.2). The medium was sterilized by passing it through a 0.45 μ m membrane filter, and then supplemented with 15% bovine serum, penicillin (400 IU/ml), streptomycin (500 μ g/ml), gentamicin (50 μ g/ml) and if necessary amikacin (125 μ g/ml).⁸ The prepared medium was dispensed into tubes filled to 80% capacity which could be used for 7-10 days at 4°C. The inoculated culture tubes were checked after one hour of incubation at 37°C for excystation and every 24 hours for development and division of the trophozoites with an inverted microscope. After the trophozoites developed, the medium was replaced every 2-4 days. When trophozoites grew well and formed a dense monolayer on the surface of the tube walls, they were dislodged by immersing the tubes in an ice-water bath for 10 min and shaking them for several seconds. 1-2 ml of this trophozoite suspension was inoculated into a tube of fresh medium.

Cryopreservation

For cryopreservation of *Giardia lamblia* trophozoites, dimethyl sulfoxide (DMSO) was used as a protectant. The trophozoites were suspended in TYI-S-33 medium containing 10% DMSO and distributed into screw-capped cryopreservation tubes and transferred to either a -70°C freezer or a liquid nitrogen tank.^{11,12,17}

RESULTS

The present study was performed on 55 fecal samples from patients with high cyst excretion. Out of 55 stool samples 26 cases had no excystation. Surprisingly, in 12 cases the cysts were observed without a cyst wall and the parasites were non-motile. Seventeen cases were found to be excysted with a rate of 4-32% (Table I).

Except for 1 case, the rest of the samples with excysted parasites were found to harbor head trophozoites after 1-7 days. One of these two cultures was discarded because of a

Table I. Excystation rate of *G. lamblia* cysts recovered from fecal samples.

Number of fecal samples	26	12	17
Excystation rate	0%	cysts without cyst wall	4-32%

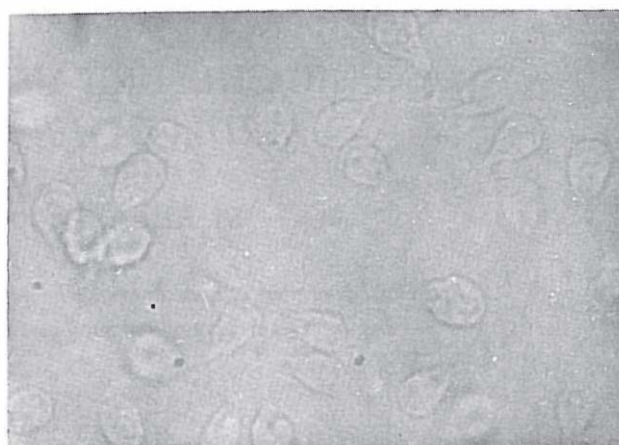


Fig. 1. *Giardia lamblia* trophozoites in culture medium (without staining).



Fig.2. *Giardia lamblia* trophozoites derived from culture medium (Giemsa staining).



Fig. 3. Right : empty *Giardia lamblia* cysts, intact cysts, and an excysted trophozoite. Left : Longitudinal division of trophozoites.

high sudden microbial contamination. The other samples had an excystation rate of 12%. After two days of excystation, groups of 2-4 motile trophozoites with incomplete divisions were observed attached to each other. Among these bizarre forms of trophozoites that were gradually degenerating, there were rare healthy trophozoites. After ten days of excystation there were 3×10^4 normal and motile trophozoites per milliliter with a normal multiplication rate and on the fourteenth day the trophozoites grew in a monolayer mode on the surface of the culture tube.

In the logarithmic phase of growth, the adherent monolayer of cells represented about 70% of the cell population. The growth of trophozoites reached a peak number of 1.4×10^6 cells/ml on the seventh day after adaptation and the generation time during the exponential phase was 10.33 hours. The formulas used for calculation of the excystation rate and the generation time are as follows:

$$\text{Excystation (\%)} = \frac{\text{ECW} + \text{PET}}{\text{ECW} + \text{PET} + \text{IC}}$$

ECW : Empty cyst wall

PET : Partially-excysted trophozoite

IC : Intact cyst³

$$\text{Generation time} = \frac{t - t_0}{3.3 \times \log \frac{Z}{Z_0}}$$

Z : Concentration of trophozoites after 24 hours.

Z₀ : Initial concentration of trophozoites

t : 24 hours after incubation of trophozoites

t₀ : Initiation time⁴

Cryopreservation of *Giardia lamblia* trophozoites in TYI-S-33 medium containing 10% DMSO was performed either in liquid nitrogen or in a freezer at -70°C. Even after thawing the organisms one year later, the trophozoites were live and motile with a survival rate of 85-90% and grew well in subculture.

DISCUSSION

There are different reports about the rate of excystation of *Giardia lamblia*. Hautus et al. reported the percentage of excystation to range from 0-90%, with rates up to 90% being observed in cysts with cystozoites initially closely applied to the cyst wall. However, in most cases (34/42) this rate was near 0-1%.⁷ They succeeded in recovering only 6 strains from 24 stool samples. In the present study which was performed on 55 stool samples, only seventeen cases were found excysted with a rate of 4-32% and from these excysted trophozoites we were able to maintain only one which was adapted in the TYI-S-33 medium. The trophozoites in Hautus' study formed a monolayer surface on the tube culture after a period ranging from 7 days to 7 weeks. In our survey the

monolayer surface was seen 14 days after hatching. Keister reported that trophozoites in the logarithmic phase could attach to the wall of the culture tube with a rate of 80-90%,¹⁰ and our study showed an attaching rate of 70%.

After Karapetian, the founder of giardia cultivation⁷, many scientists have since investigated culturing *Giardia lamblia* in different media such as HSP₁, HSP₂¹⁵, TPS₁¹⁸ and TYI-S-33.⁴

Kasprzak reported the generation time of 20 strains of *G. lamblia* to range from 8.1 ± 0.2 to 31.2 ± 2.6 in the TPS₁ medium. This period decreased markedly (from 8.1 ± 0.2 to 15.6 ± 0.5) in BI-S-33 supplemented with bovine bile.⁹ Addition of bovine bile to TYI-S-33 medium also reduced the generation time. The lowest generation time was obtained with fresh bovine bile (7.5 ± 0.5 hours).⁵ Lu et al. reported the generation time in the TYI-S-33 medium to be 15 ± 2 hours.¹² In our study the calculated generation time was about 10.33 hours.

After reviewing the literature and our own results, we believe that *G. lamblia* is a very fastidious organism with an excystation rate, generation time and media adaptation which is related to differences between strains. Therefore, the development of defined medium without undefined biological products is needed to advance the axenic culture systems of giardia.

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