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Optimized Conditions for *In Vitro* High Density Encystation of *Giardia lamblia*

HONG, WOOK-SUN, KYONG-JOO KIM, AND KISAY LEE*

Department of Environmental Engineering and Biotechnology, Myongji University, Yongin, Kyongki 449-728, Korea

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Abstract *Giardia lamblia*, a waterborne parasitic protozoa causing diarrhea and gastroenteritis, is transmitted to humans from untreated and treated water in the form of cysts. The ingestion of *G. lamblia* cysts is followed by the excystation of the cysts to trophozoites and subsequent colonization of the upper small intestine. In this study, the *in vitro* conditions for *G. lamblia* encystation were investigated to enhance the efficiency of cyst conversion and the resulting cyst density. The trophozoite of *G. lamblia* was cultivated to the late exponential growth phase, resulting in a high density of over 6×10^7 cells/ml. The effects of pH, bile content, and induction time were evaluated: A cyst conversion of over 25% and 10^7 cysts/ml were routinely obtained using the optimized encystation conditions including a slightly alkaline pH, 10 to 15 mg/ml of bile concentration, and 48–50 h of induction time.

Key words: *Giardia lamblia*, cysts, trophozoites, *in vitro* encystation

The protozoan parasite, *Giardia lamblia*, is a worldwide waterborne pathogen that causes diarrhea and gastroenteritis in humans and animals [1]. It is transmitted to humans in the form of cysts through untreated water as well as treated drinking water [9]. The ingestion of *G. lamblia* cysts is followed by the excystation of the cysts to trophozoites and subsequent colonization of the upper small intestine. A trophozoite is a pear-shaped flagellate with a bilateral symmetry (8–14 μm) and four pairs of flagella. Before being released into the environment from the host, some trophozoites are transformed in the distal ileum and/or the large intestine into cysts (encystation), a form that is resistant against harsh environmental conditions. These cysts are 6–8 μm ovals, have four nuclei [8], and are also covered with a well-defined filamentous wall which makes them impervious to inactivation by usual drinking water disinfectants like chlorine [4, 9, 14].

*Corresponding author
Phone: 82-31-330-6392; Fax. 82-31-337-2902,
E-mail. kisay@wh.myongji.ac.kr

Since their presence in water has led to frequent outbreaks of giardiasis in many countries, methodologies for monitoring and removing this protozoa from the water supply are of great public health concern [3, 9, 14]. A large quantity of cysts is required for studies to develop methods for detecting and monitoring *G. lamblia* in water samples, examining and improving the performance of installed water treatment facilities, and searching for better disinfectants to protect drinking water from this parasite.

However, at present, most scientists and engineers in this field rely on the direct recovery and isolation of cysts from contaminated water or infected host for their supply of cysts [2, 3, 13], because effective methods of cultivation and the *in vitro* encystation of *G. lamblia* have not yet been established. Furthermore, the efficiency of cyst conversion and the resulting cyst concentrations are poor. A widely used procedure for inducing encystation *in vitro* is to expose trophozoites to a high-bile condition, thereby mimicking the harsh environment in the large intestine where cyst formation is triggered *in vivo* [6, 8, 10]. The previously reported encystation efficiency would appear varied among different *Giardia* species. For *G. lamblia*, a cyst conversion of 2 to 50% and a resulting cyst density of 10^5 to 10^6 cysts/ml have been generally obtained on a laboratory scale [5, 6]. Accordingly, we attempted to optimize some of the *in vitro* encystation conditions for *G. lamblia* to enhance the cyst concentration. The effects of pH, bile concentration, and induction time on the cyst conversion and the resulting cyst density were examined.

Cultivation of *G. lamblia* Trophozoites

Trophozoites of *G. lamblia*, isolated from a Korean patient with chronic symptomatic giardiasis, were provided by Prof. S.-J. Park [11] at the College of Medicine, Yonsei University. The cells were routinely cultivated in a filter-sterilized Diamond TYI-S-33 medium [7] supplemented with 10% adult bovine serum and 0.5 mg/ml of bovine bile at pH 7.1. When the attached cells formed a confluent monolayer, subcultures were made in 15×125 mm (working volume 14 ml) screw-capped borosilicate glass tubes at 37°C. These subcultures were then chilled in an ice bath to

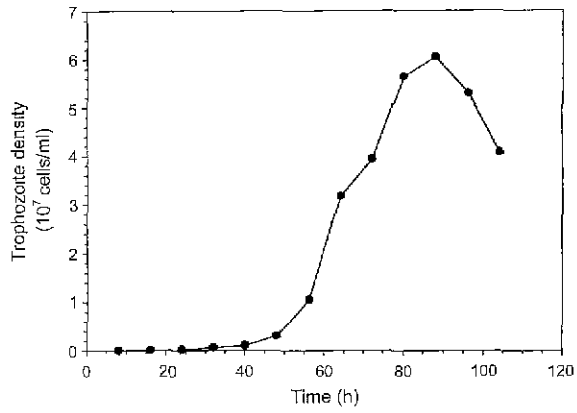

Fig. 1. Growth curve of *Giardia lamblia* trophozoite in TYI-S-33 medium with 0.5 mg/ml of bovine bile at pH 7.1 and 37°C.
The optical density at 600 nm during the cultivation was measured after dislodging the cells in the confluent monolayer and was correlated with the cell density by microscopic counting.

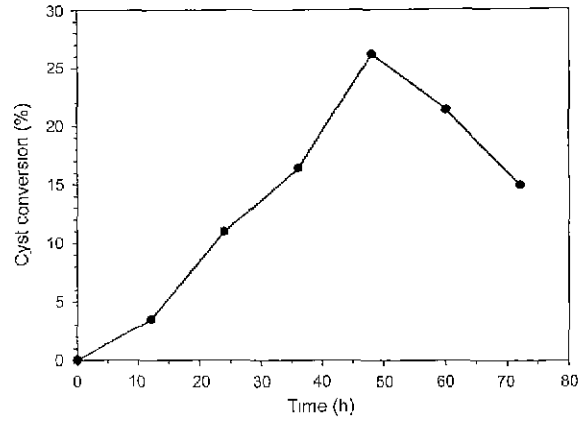

Fig. 2. Change of cyst conversion with different induction times in an encystation medium with 10 mg/ml of bovine bile at pH 7.8 and 37°C.
Trophozoites in the late exponential growth phase in a confluent monolayer with approximately 6×10^7 cells/ml were used.

dislodge the cells and 2% of the inoculum was transferred into a tube with fresh medium.

To determine the optimal incubation time to obtain a high cell density in the confluent monolayer, after pouring off the unattached cells and the spent medium, the optical density at 600 nm during the cultivation was measured by resusperding the cells homogeneously in phosphate buffered saline, pH 7.4 at 4°C. The optical density was correlated with the cell population by counting the trophozoite cells with a hemocytometer under a phase contrast microscope after iodine staining. Figure 1 shows the growth curve of *G. lamblia* trophozoites. There was a lag phase for the initial 50 h, followed by an exponential growth phase up to 90 h where a cell density of over 6×10^7 cells/ml was obtained.

Encystation of Trophozoites into Cysts

After the trophozoites were grown in the confluent monolayer for 80 to 90 h (approximately 6×10^7 cells/ml) in a normal TYI-S-33 medium containing 0.5 mg/ml of bovine bile at pH 7.1, they were transferred to an encystation medium containing 10 mg/ml of bovine bile with a slightly alkaline pH. This high bile concentration for the induction of encystation was to mimic the physiological condition of the distal ileum and large intestine of a human host, where the natural differentiation into cysts is initiated [6, 8, 10]. After incubation in the high-bile encystation medium for a given time at 37°C, the cells were then transferred back to the normal TYI-S-33 medium for another 24 h. The cells were dislodged by chilling at 4°C, the medium removed, and the undifferentiated trophozoites were then extracted by hypotonic lysis in distilled water. The remaining cysts were enumerated under a microscope after vital staining [13]. It has previously been demonstrated that this procedure of *in vitro* encystation produces genetically identical clones of both cysts and trophozoites [15].

Since the residence time in a human host is assumed to be about one day, 24 h of encystation in a high bile medium has usually been recommended by other researchers [5, 8, 15] however, this may not be an optimal period for achieving maximum cyst formation. To determine an optimal induction time for encystation, the efficiency of the cyst conversion was measured at various incubation times in a high bile medium with 10 mg/ml of bovine bile at pH 7.8. As shown in Fig. 2, the conversion to cysts was best obtained after 50 h of induction, where the cyst conversion was 27%, corresponding to about 1.6×10^7 cysts/ml.

The role of bile in the encystation medium is due to the fact that cyst formation is induced in a lipid-poor environment in the host organs and a high concentration of bile salts inhibits cholesterol uptake by trophozoites [5, 10]. Therefore, a bile-rich condition in the encystation medium stresses the trophozoites, thereby favoring encystation. To identify the best bile concentration, encystation was carried out for 48 h in encystation media with different bile contents at pH 7.8 (Fig. 3). The best conversion of over 25% was obtained with a bile concentration ranging between 10–15 mg/ml. The lower conversion with 20 mg/ml of bile was due to an extremely high bile concentration which was unfavorable to the survival of the trophozoites themselves (data not shown).

It is also recognized that a slightly alkaline pH favors the encystation of *Giardia* [8, 10]. Figure 4 shows the cyst conversion with various pHs, while other encystation conditions such as bile and incubation time were fixed at 10 mg/ml and 48 h, respectively. The pH had a great influence on the cyst conversion and the optimal pH was identified to be between 7.7 to 7.9, in which about 27% of cyst was converted. The lower conversion at below pH 7 and above pH 8.5 was due to decreased survival of the trophozoites themselves in such pH regions (data not shown).

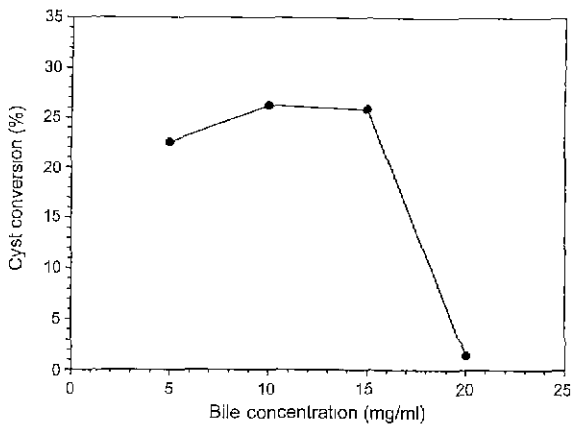

Fig. 3. Effect of bile concentration in the encystation medium on cyst conversion of *G. lamblia* after 48 h of incubation at pH 7.8 and 37°C.

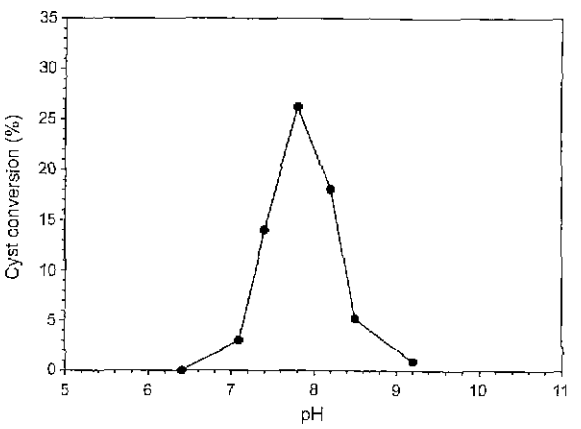

Fig. 4. Effect of encystation medium pH on cyst conversion of *G. lamblia*

The other encystation conditions were fixed at 10 mg/ml of bile and 48 h of incubation time

In summary, the procedure for the *in vitro* encystation of *G. lamblia* was improved by identifying optimal conditions for a high cyst conversion rate and cyst concentration, in contrast to the conventional procedures which simply mimic the physiological environment inside a human host. Accordingly, encystations were carried out using a high density of trophozoites in the late exponential phase of growth, together with optimized encystation conditions, including a slightly alkaline pH. 10 to 15 mg/ml of bile concentration, and 48 to 50 h of induction time. These conditions routinely produced a cyst conversion of more than 25% and 10^7 cysts/ml.

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