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▣ ORIGINAL ARTICLE

Efficient Liquid Media for Encystation of Pathogenic Free-Living Amoebae

Hae-Jin Sohn[1], Heekyoung Kang[1], Ga-Eun Seo[1], Jong-Hyun Kim[2], Suk-Yul Jung[3], Ho-Joon Shin[1,*]

[1]Department of Microbiology, Ajou University School of Medicine, and Department of Biomedical Science, Graduate School of Ajou University, Suwon 16499, Korea; [2]Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Korea; [3]Department of Biomedical Laboratory Science, Molecular Diagnostics Research Institute, School of Health and Medicine, Namseoul University, Cheonan 31020, Korea

Abstract: Pathogenic *Naegleria fowleri*, *Acanthamoeba castellanii*, and *Acanthamoeba polyphaga*, are distributed worldwide. They are causative agents of primary amoebic meningoencephalitis or acanthamoebic keratitis in humans, respectively. Trophozoites encyst in unfavorable environments, such as exhausted food supply and desiccation. Until recently, the method of *N. fowleri* encystation used solid non-nutrient agar medium supplemented with heat-inactivated *Escherichia coli*; however, for the amoebic encystment of *Acanthamoeba* spp., a defined, slightly modified liquid media is used. In this study, in order to generate pure *N. fowleri* cysts, a liquid encystment medium (buffer 1) modified from Page's amoeba saline was applied for encystation of *N. fowleri*. *N. fowleri* cysts were well induced after 24 hr with the above defined liquid encystment medium (buffer 1). This was confirmed by observation of a high expression of differential mRNA of *nfa1* and actin genes in trophozoites. Thus, this liquid medium can replace the earlier non-nutrient agar medium for obtaining pure *N. fowleri* cysts. In addition, for cyst formation of *Acanthamoeba* spp., buffer 2 (adjusted to pH 9.0) was the more efficient medium. To summarize, these liquid encystment media may be useful for further studies which require axenic and pure amoebic cysts.

Key words: *Naegleria fowleri*, *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, encystation, encystment medium

INTRODUCTION

Pathogenic free-living amoebae, *Naegleria fowleri*, *Acanthamoeba castellanii*, and *Acanthamoeba polyphaga*, are distributed worldwide, and are the known causative agents of primary amoebic meningoencephalitis (PAM) or acanthamoebic keratitis (AK) in humans, respectively [1]. PAM cases are associated with recreational activities in freshwater, whereas AK cases have become a concern in people wearing contact lenses. The ubiquitous distribution in nature of these free-living amoebae shows 2 distinct forms in their life-cycle: trophozoites and cysts (sometimes flagellate in *Naegleria*). In unfavorable environments, such as exhausted food supply, desiccation, and low temperature, amoebic trophozoites are known to undergo encystation. The cysts are characterized by a round shape and the presence of 2 cyst walls (endo- and ectocyst), especially distinct in the wrinkled ectocyst in *Acanthamoeba* spp. [2]. The encystation is an important event for the survival, resistance, and ability of disease induction of these free-living amoebae [3-6].

Until recently, a useful method of *N. fowleri* encystation was the use of solid non-nutrient agar medium supplied with heat-inactivated *Escherichia coli*. The solid medium has a limitation for cultivation and harvesting axenic and massive amoebic cysts. Current studies in *Naegleria* infection indicate that the encystation of *N. fowleri* trophozoites is required to study the pathophysiology, pathogenic mechanisms, immunology, and genomics. On the other hand, since a defined liquid media for the encystment of *Acanthamoeba* spp. has been established [6], many researchers have lately applied acanthamoebic encystation with a little modification [7-10].

To obtain a large amount of pure *N. fowleri* cysts (or precysts), a liquid encystment medium modified from Page's amoeba saline [1] was applied on encystation of *N. fowleri* trophozoites. In addition, the efficient usage of 2 liquid encystment media generally used for the cyst formation of *Acanthamoeba* spp. [6,11] was compared.

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* Corresponding author (hjshin@ajou.ac.kr)

MATERIALS AND METHODS

Free-living amoebae cultivation

Trophozoites of *N. fowleri* (Cater NF69 strain, ATCC No. 30215, Manassas, Virginia, USA) were axenically cultured at 37℃ in Nelson's medium containing 10% fetal bovine serum [12], and *A. castellanii* and *A. polyphaga* were axenically cultured at 30℃ in PYG medium [13].

Encystation of free-living amoebae

Cysts or precysts (did not fully showed the 2-distinct cyst walls) of *N. fowleri*, *A. castellanii*, and *A. polyphaga* were induced by cultivating on 3 kinds of encystment media (buffer 1: 120 mM NaCl, 0.03 mM $MgCl_2$, 1 mM $NaHPO_4$, 1 mM KH_2PO_4, 0.03 mM $CaCl_2$, 0.02 mM $FeCl_2$, H 6.8; buffer 2 [11]: 95 mM NaCl, 5 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$, 20 mM Tris-HCl, pH 9.0; buffer 3 [6]: 100 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 20 mM 2-amino-2-methyl-1,3-propanediol, pH 7.6) (Table 1). Amoebic trophozoties (approximately 2×10^6 cells) were washed with PBS (pH 7.4) twice, and incubated in 24-well plates with 5 ml of each medium, at 30℃ or 37℃. Using an optical microscope (Olympus, Shinjuku, Tokyo, Japan), the morphological changes were observed after encystation, and final cysts were re-cultured with fresh Nelson or PYG media, in order to observe the recovered trophozoites.

Table 1. Compositions of encystation media for *Naegleria* (buffer 1), *Acanthamoeba* spp. (buffers 2 and 3), and Page's amoeba saline

Buffer 1. *Naegleria* Encystment media (pH6.8)	
120 mM	NaCl
0.03 mM	$MgCl_4$
1 mM	Na_2HPO_4
1 mM	KH_2PO_4
0.03 mM	$CaCl_2$
0.02 mM	$FeCl_2$
Buffer 2. *Acanthamoeba* Encystment media (pH9.0)	
95 mM	NaCl
5 mM	KCl
8 mM	$MgSO_4$
1 mM	$NaHCO_3$
0.4 mM	$CaCl_2$
20 mM	Tris-Cl (Ph9.0)
Buffer 3. *Acanthamoeba* Encystment media (pH7.6)	
0.1 mM	KCl
8 mM	$MgSO_4$
0.4 mM	$CaCl_2$
20 mM	2-amino-2-methlyl-1, 3-propanediol
Page's amoeba saline (pH6.8-7.9)	
2 mM	NaCl
0.03 mM	$MgSO_4$
0.03 mM	$CaCl_2$
1 mM	Na_2HPO_4
1 mM	KH_2PO_4

Differential mRNA expression from trophozoites and cysts of free-living amoebae

Total RNA from trophozoites and cysts (or precysts) of *N. fowleri*, *A. castellanii*, and *A. polyphaga* were prepared using an isolation kit Rnasymini kit (QIAGEN, Hilden, Germany); cDNAs were synthesized from 10 μg of total RNA using cDNA Synthesis kit (Invitrogen, Carlsbad, California, USA). RT-PCR was performed using specific primer for *nfa1* and *nf-actin* genes for *Naegleria*, and *actin* and *atg8* genes for *Acanthamoeba* [5,14, 15]. The reference genes were the *p*3 gene (*N. fowleri*-specific chromosomal DNA sequence; forward 5′GCTATCGAATGGATTCAAGC and reverse 5′CACTACTCGTGGAAGGCTTA) and *18S r*RNA gene [5]. PCR condition was 95℃ for 5 min, 30 cycles at 95℃ for 1 min, 50-55℃ for 1 min, 72℃ for 1 min, and final extension for 10 min at 72℃.

RESULTS

Morphological observation of encysted amoebae

Trophozoites of *N. fowleri* cultured with buffer 1 showed the rounding morphological change at 6 hr, and precysts and cysts were observed at 24 hr. After 48 hr cultivation, a large proportion of *N. fowleri* trophozoites were encysted (Fig. 1). On culturing the *N. fowleri* trophozoites in buffer 2, the morphological changes (such as rounding), precysts and cysts were induced later than seen in buffer 1 (Fig. 1). On all occasions, the induced precysts and cysts at 48 hr were recovered into active trophozoites in fresh Nelson's medium (Fig. 1). As a control group, *N. fowleri* trophozoites cultured in distilled water (DW) did not encyst fully. In addition, when they were incubated with staurosporine, a reagent known to induce cell-death, *N. fowleri* trophozoites transformed to their round forms, but live trophozoites could not be recovered in Nelson's medium (Fig. 1).

Trophozoites *A. castellanii* cultured with buffer 2 and 3 showed rounding at day 1, and precysts and cysts were well observed at day 2. After cultivation for 3 days, a large proportion of *A. castellanii* trophozoites were encysted (Fig. 2). Culturing *A. castellanii* trophozoites in buffer 2, the induction of precysts and cysts was better than those of buffer 3 (Fig. 2). On

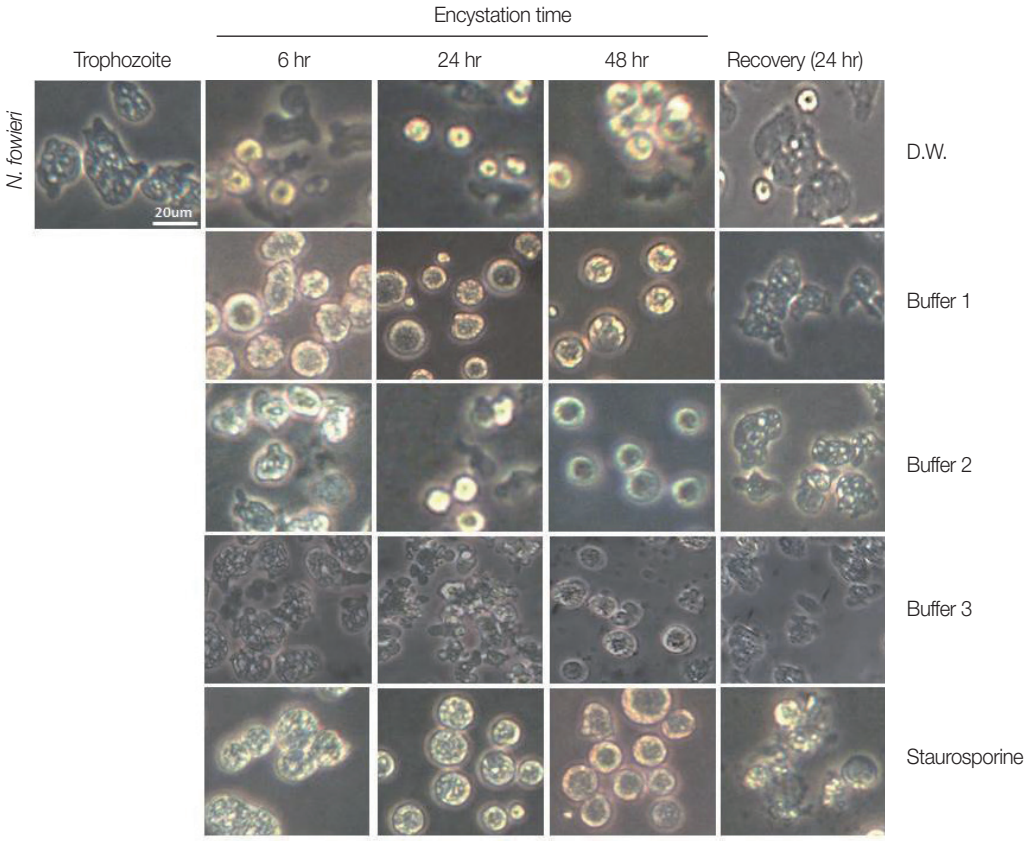

Fig. 1. Morphological changes of *N. fowleri* trophozoites into precysts (or cysts) cultured with encystation media for 6, 24, and 48 hr. Buffer 1 is an effective medium. Staurosporine induced rounding, but trophozoites were not recovered in Nelson's medium.

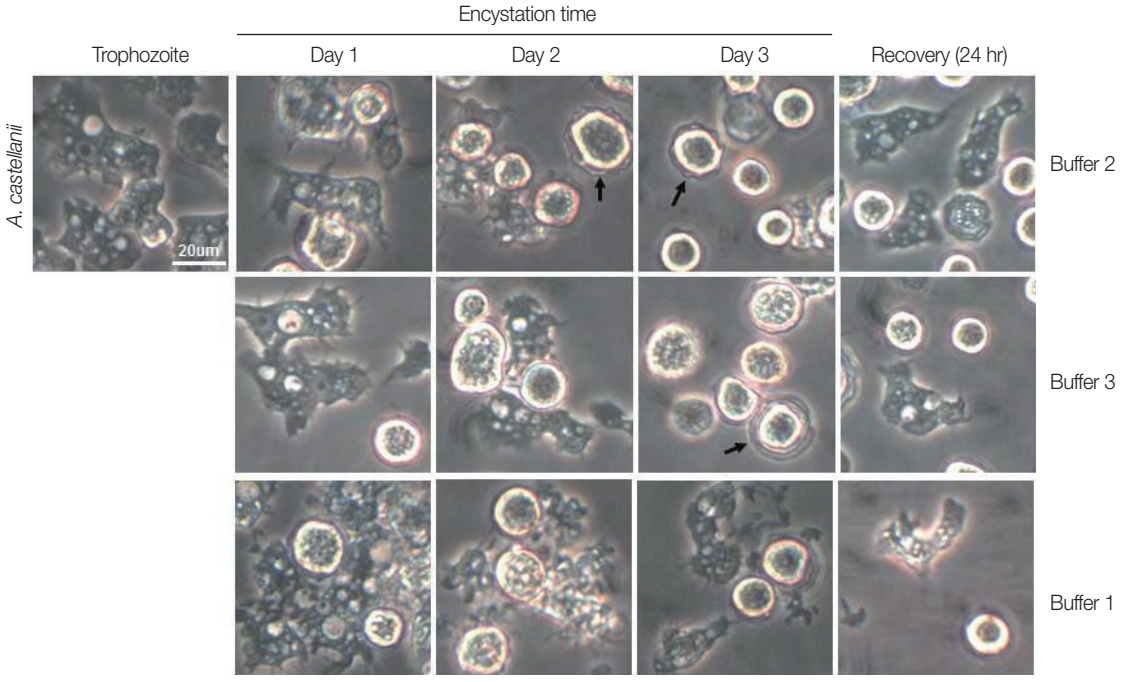

Fig. 2. Morphological changes of *A. castellanii* trophozoites into precysts (or cysts) cultured in encystation media for 1, 2, and 3 days. The effective medium is buffer 2. Cysts recovered to trophozoites in PYG medium. Arrows indicate mature cysts.

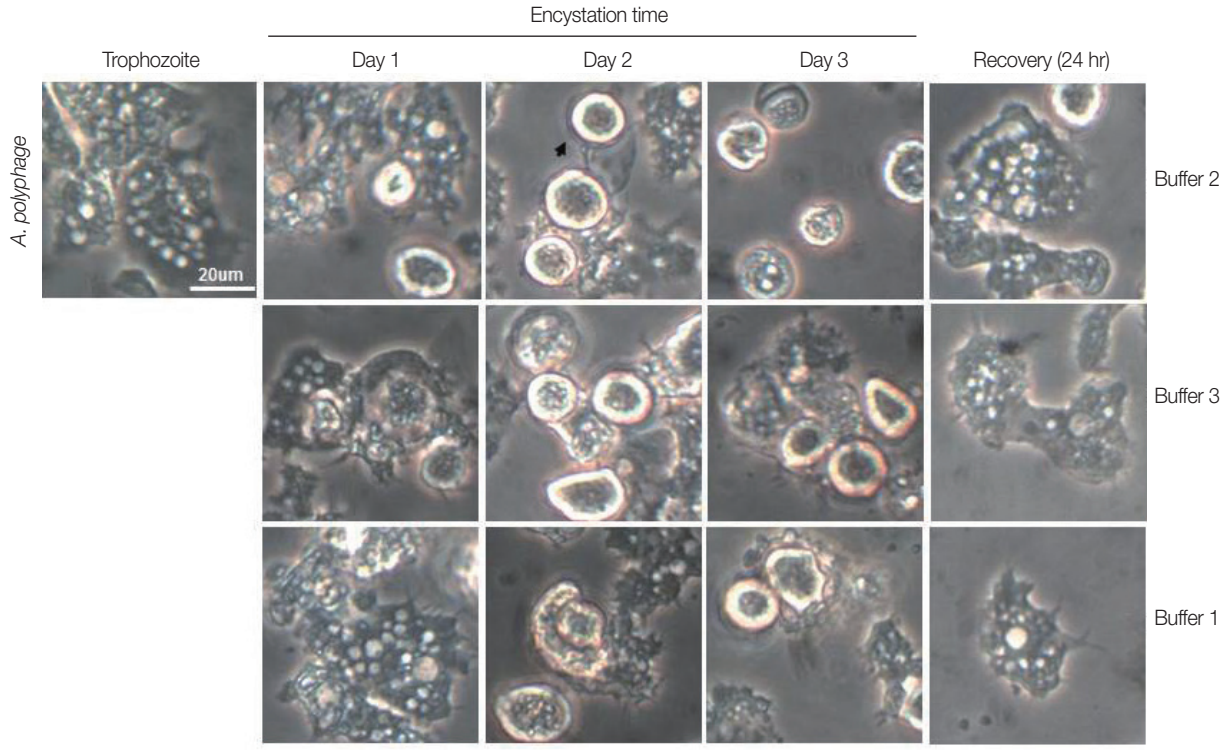

Fig. 3. Morphological changes of *A. polyphaga* trophozoites into precysts (or cysts) cultured in encystation media for 1, 2, and 3 days. Buffer 2 is an effective medium. Cysts recovered to trophozoites in PYG medium.

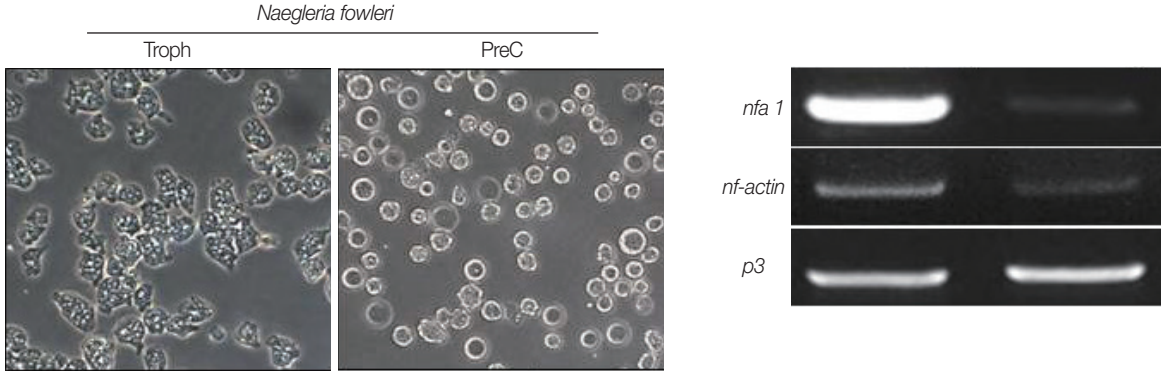

Fig. 4. The mRNA expressions of *nfa1* and *nf-actin* genes on *N. fowleri* trophozoites (Troph), precyts, and cysts (PreC) at 48 hr post cultivation with buffer 1. The *p3* mRNA expression is used as the control.

all occasions, induced precysts and cysts for 3 days recovered into active trophozoites in fresh PYG medium (Fig. 2). Furthermore, *A. castellanii* trophozoites cultured with buffer 1 (shown the well encystation in the case of *N. fowleri*) did not encyst fully (Fig. 2). When encystation was continued for 7 days, similar results were observed, although the number of cysts decreased in accordance with incubation periods (data not shown). In addition, in the case of *A. polyphaga*, the encystation results were similar to those of *A. castellanii* (Fig. 3).

Differential mRNA expression in trophozoites and cysts of amoebae

In the results of differential mRNA expression between trophozoites and cysts (precyst) of *N. fowleri*, the *nfa1* gene was highly expressed in the trophozoites (Fig. 4). In addition, the *nf-actin* gene on trophozoites was also more expressed than in cysts (Fig. 4). In the differential mRNA expression of acanthamoebic actin gene, over-expression was observed in trophozoites of *A. castellanii* and *A. polyphaga* (Fig. 5). In contrast,

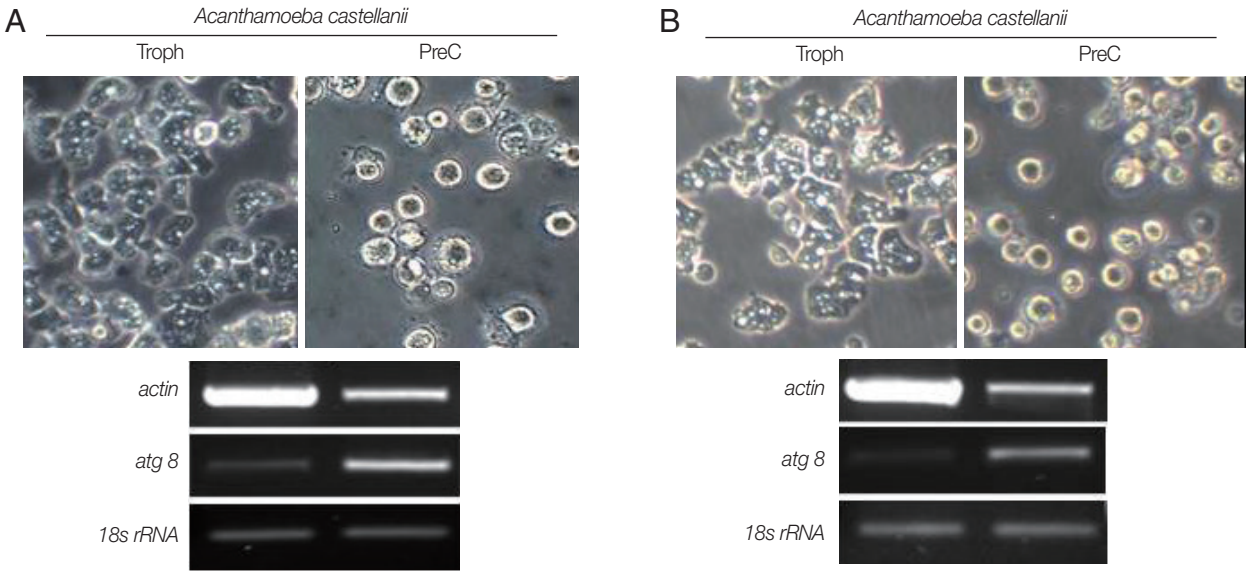

Fig. 5. The mRNA expressions of *actin* and *atg8* genes on *A. castellanii* (A) and *A. polyphaga* (B) trophozoites, precysts and cysts. The *18S* rRNA mRNA expression is used as the control.

the *atg8* gene which is associated with cyst formation in *Acanthamoeba* spp. [11] was expressed more in cysts (Fig. 5).

DISCUSSION

N. fowleri, known as "brain-eating amoeba", causes acute PAM in children and young adults with a history of diving in freshwater and recreational activities in amoebae-contaminated water in the hot summer [2,16]. In these latter days, death due to PAM is rearing its ugly head as a Neglected Tropical Disease (NTD) in countries of Asia, such as India, Pakistan, Taiwan, Thailand, and Vietnam [17]. In addition, the AK human case from contact lens wearers has been increasing worldwide [18].

In recent many studies on free-living amoebae, such as *N. fowleri* and *A. castellanii*, the encystation of amoebic trophozoite is required to study the pathophysiology, pathogenic mechanisms, immunology, and genomics. For the amoebic encystment of *Acanthamoeba* spp., in previous studies, a defined and slightly modified liquid media has been used [6,9,10]. In *N. fowleri* encystation, however, the solid non-nutrient agar medium supplemented with heat-inactivated *E. coli* has been used, in spite of the possible contamination of *E. coli* proteins and smaller numbers of harvested cysts [4,19,20].

In this study, to obtain a large amount of pure *N. fowleri* cysts (or precysts), a liquid encystment medium modified from Page's amoeba saline (named as the encystmemt buffer 1) was applied on *N. fowleri* encystation. As the results of *N. fowleri* encystation with buffer 1, amoebic cysts were well induced at 48 hr post incubation. And then, the induced cysts at 48 hr were well recovered into active trophozoites in fresh Nelson's medium. In addition, the above morphological encystation was confirmed by observing the differential mRNA expression of the pseudopodia-specific *nfa1* gene [14] and the *nf-actin* gene [15] between trophozoites and cysts of *N. fowleri*. On the present results, the *nfa1* and *nf-actin* gene on trophozoites was also more expressed than in cysts. These results proved the encystation of *N. fowleri* trophozoites. Thus, the present method used a liquid encystment medium may replace the previous method of *N. fowleri* encystation used a solid non-nutrient agar medium with heat-inactivated *E. coli*.

On the morphological findings of *Acanthamoeba* encystation, buffer 2 (modified to pH 9.0) was also a more effective agent in this study, which was mainly used for many studies [6,8,9,11]. In addition, the present results were confirmed by the differential mRNA expression between trophzoites and cysts. The over-expression of actin gene was observed in trophozoites of *A. castellanii* and *A. polyphaga*. In contrast, the *atg8* gene was expressed more in cysts, as which is associated with cyst formation in *Acanthamoeba* spp. [11]. The above results indicate that buffer 2 is better for the encystation of *A. castellanii* and *A. polyphaga* trophozoites.

Finally, 2 liquid encystment media, buffer 1 for *Naegleria* and buffer 2 for *Acanthamoeba* may be useful for further studies which require axenic and pure amoebic cysts.

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CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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