# CHARACTERIZATION OF CYST AND TROPHOZOITE PROTEINS OF ENVIRONMENTAL ISOLATES OF *ACANTHAMOEBA CASTELLANII* BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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**Abstract.** *Acanthamoeba castellanii* has been known to possess pathogenic properties, such as acanthamoebic keratitis and granulomatous amoebic encephalitis. The role of proteases and proteins in the pathogenesis of these infections is still poorly understood. As *Acanthamoeba* sp is a ubiquitous protozoon found in the natural environment they can potentially cause human infections. This study characterized cyst and trophozoite proteins of 3 environmental *A. castellanii* isolates in comparison with a clinical isolate, ATCC 50492. The latter and environmental IMU1 isolate had 100% genotype identity with *A. castellanii* and demonstrated protein spots with higher molecular weights (> 95 kDa) at relatively higher isoelectric values (> pI 7.00) compared to the two other environmental isolates (IMU4 and IMU5) that had 99% genotype identity to *A. castellanii* based on 16 S rDNA sequence. Thus such trophozoite proteins may be involved with the parasite's ability to cause acanthamoebic keratitis.

**Keywords:** *Acanthamoeba castellanii*, cyst, trophozoite protein, two-dimensional gel-electrophoresis

## INTRODUCTION

*Acanthamoeba* sp is a ubiquitously distributed free-living amoeba found in various environments and has recently become well recognized as one of the microbes of pathogenic importance (Mergeryan, 1991; Rivera *et al*, 1991; Rodriguez-Zaragosa, 1994; Booton *et al*, 2004; Khan, 2006). As *Acanthamoeba* sp possesses pathogenic potential, research is increasingly being carried out to understand the mechanisms and processes involved in pathogenicity (Khan *et al*, 2000; Maciano-Cabral and Cabral, 2003; Khan, 2006; Siddiqui *et al*, 2011).

The majority of *Acanthamoeba* isolates do not exhibit pathogenicity towards humans, and only under certain conditions the human host becomes susceptible to pathogenic strains of *Acanthamoeba*. Culbertson *et al* (1958, 1959) first promulgated the suggestion that *Acanthamoeba* sp has pathogenic potential as evidenced by cell-mediated cytotoxicity in monkey kidney cells, together with the demonstration of

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infection in monkeys and mice. However it was only in the 1970's that Nagington *et al* (1974) first reported the incidence of acanthamoebic keratitis, and during the past 30 years, reports of acanthamoebic keratitis and granulomatous acanthamoebic encephalitis have increased worldwide (Khan, 2006). *Acanthamoeba* has also been reported to infect the gastrointestinal tract in a patient with pyloric perforation (Thamprasert *et al*, 1993).

One of the various factors involved in the pathogenesis of acanthamoebic infections is the production and secretion of glycoproteins and proteases by *Acanthamoeba* sp. Proteases have been well documented as being a major virulence factor in the pathogenesis of various diseases and are involved in *Acanthamoeba*-mediated host tissue destruction and digestion of phagocytozed food (Khan, 2006). Amoebic proteolytic enzymes include serine proteases (Hadas and Mazur, 1993; Mitra *et al*, 1995; Moon *et al*, 2008), contact-dependent metallo-proteases (Khan *et al*, 2000), elastases (Hadas and Mazur, 1993) and cytotoxic proteases induced by mannose-mediated adhesion (Leher *et al*, 1998), the latter involving mannose-binding protein (MBP), which plays an important role in mediating the binding of *Acanthamoeba* to target/host cells (Leher *et al*, 1998). There is a relatively higher serine protease activity reported in clinical than environmental *Acanthamoeba* isolates (Khan, 2006).

Proteomics approaches, such as two-dimensional gel-electrophoresis (2D-GE) have recently been used to identify proteins involved in or are present during encystation of *A. castellanii (*Bouyer *et al,* 2009; Leitsch *et al,* 2010). Although progress has been made in the study of the encystation process of *A. castellanii* (Chávez-Munguía *et al*, 2005; Bouyer *et al*, 2009), the identities of proteins and enzymes in the cyst and trophozoite stages of *Acanthamoeba* related to their differentiation remain to be studied in order to understand the cellular differentiation and morphogenesis. In addition, the role of *Acanthamoeba* proteases in pathogenesis needs further elucidation. Specific protein targets identified from trophozoite and cyst stages from these studies could also serve as potential targets for the development of new therapeutics as current drugs used for treatment of granulomatous amoebic encephalitis, such as pentamidine and ketoconazole, are still not totally effective (Marciano-Cabral and Cabral, 2003).

In this study, 2D-GE was used to compare the protein profiles of 3 environmental isolates with a clinical isolate in order to identify candidate proteins of interest that could be further studied to determine their role in pathogenesis or as drug candidates.

## MATERIALS AND METHODS

### *Acanthamoeba* isolates

Four isolates of *A. castellanii* were obtained from a previous study conducted by Chan (2009), comprising of a clinical isolate, ATCC 50492, and 3 environmental isolates (IMU1, IMU4 and IMU5). Of the 3 environmental isolates, IMU1 has 100% genetic identity to *A. castellanii* whereas IMU4 and IMU5 have 99% identity based the 18 S rDNA sequence *Acanthamoeba* sp (Chan, 2009). All isolates demonstrate group II morphology and are T4 isolates.

### Growth of *Acanthamoeba* isolates

All *Acanthamoeba* isolates were cultured *in vitro* in peptone-yeast-glucose (PYG) medium supplemented with 10% fetal bovine serum (FBS), (Gibco, Big

Cabin, UK) (complete culture medium) at 26-28ºC (room temperature) in 75 $cm^2$ tissue culture flasks (Nunc; Thermoscientific, Rochester, NY) with filter caps. All culture and subculture were conducted axenically in a Class II biological cabinet (NuAire, Plymouth, MN).

**Growth of *Acanthamoeba* trophozoites and encystation**

Each flask was seeded with ~ 1 x $10^6$ cells in 15 ml complete culture medium and growth of the parasites was monitored daily under an inverted microscope. For each isolate, 10 flasks of cultures were grown for 2 days. Flasks containing mainly trophozoites (> 95% or more) were harvested for protein extraction and quantitation.

Encystation of *Acanthamoeba* isolates was induced using the Band encystation medium consisting of 3.2 mM $MgCl_2$, 0.36 mM $CaCl_2$, and 0.25 M NaCl. From 1 x $10^6$ - 1 x $10^7$ *Acanthamoeba* cells were cultured in 15 ml of Band encystation medium for 5-7 days and then cysts (> 95%) were harvested for protein extraction and quantitation.

**Harvesting of *Acanthamoeba* sp trophozoite and cyst proteins**

Once cells were subcultured, trophozoites were harvested on the second day and counted using a hemocytometer. Trophozoites were ultrasonicated 4 times at 0.5 amplitude for 15 seconds each time (Sartorius AG, Goettingen, Germany) and cell lysates were then centrifuged (Eppendorf, Humburg, Germany) at 4,000*g* for 20 minutes at 4ºC. Supernatants were stored at -80ºC until used.

Cysts were collected using a cell scraper (Greiner-Bio One, Monroe, NC) in 4 ml of phosphate-buffered saline (PBS), pH 7.4, and the cyst suspension was placed in a 5 ml Bijou bottle with the addition of 1 ml of protein inhibitor cocktail (Roche, Indianapolis, IN). Cell suspension was ultrasonicated once at 0.5 amplitude for 30 seconds (Sartorius AG, Goettingen, Germany) in order to disrupt all trophozoites. The number of cysts was counted using a hemocytometer and cysts were collected by centrifugation at 800*g* for 10 minutes and the pellet resuspended in 1 ml of PBS and homogenized using glass beads (Tomy Beads Cell Disrupter - Micro Smash MS-100, Tokyo, Japan) 15 times at 30 seconds each time with a 10-minute interval on ice. Cell lysate was then centrifuged (Eppendorf, Hamburg, Germany) and supernatant stored at -80ºC until used.

Quick Start™ Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) was used to determine trophozoite and cyst protein concentrations, using different concentrations of bovine serum albumin as standards.

**2D-GE**

*Acanthamoeba* trophozoite and cyst protein extracts were purified with a 2D clean-up kit (GE Healthcare, Pittsburg, PA), performed on ice according to the manufacturer's protocol, prior to isoelectric focusing (IEF) step. Each *Acanthamoeba* sample was adjusted to 750 g/ml of protein.

A dry immobilized pH 3-10 gradient strip (IPG; Immobiline Dry Strip; 11 cm; GE Healthcare, Pittsburg, PA) was rehydrated overnight at room temperature together with purified *Acanthamoeba* protein sample (Bio-Rad Laboratories, Hercules, CA). IEF process was performed for a total of 5.5 hours as follows: rehydration, 12 hours; conditioning, 250 V for 20 minutes, linear mode; voltage ramping, 8,000 V for 2.5 hours, linear mode; focusing, 8,000 V until 20,000 V/hr (2.5 hours); and

Table 1
Number of stage-specific and shared protein spots seen in the 4 *Acanthamoeba* isolates.

| Isolate | Trophozoite stage | | Cyst stage | | Shared spots |
|---|---|---|---|---|---|
| | Total no. of spots | No. of specific spots | Total no. of spots | No. of specific spots | |
| ATCC 50492 | 86 | 76 | 45 | 35 | 10 |
| IMU1 | 69 | 59 | 21 | 11 | 10 |
| IMU4 | 37 | 30 | 16 | 9 | 7 |
| IMU5 | 23 | 21 | 16 | 14 | 2 |

hold, 500 V was maintained until the end of the process. The IBG strip was stored at -80ºC overnight before conducting the second dimension separation.

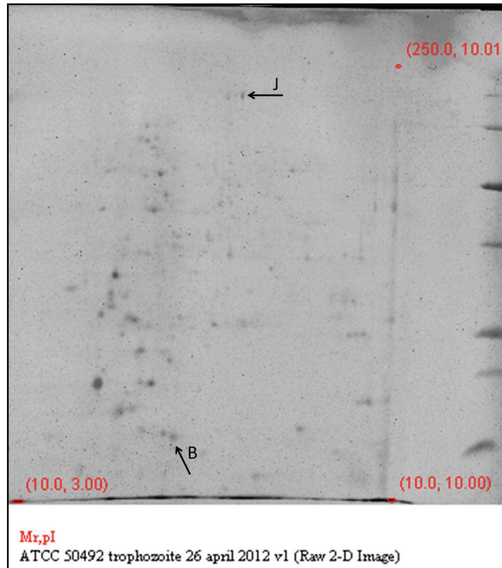
A maxi 12% resolving gel (16.5 cm x 17.5 cm, W x H) was prepared using Scie-Plas TV400Y system (Scie-Plas, UK). IPG strips were equilibrated at room temperature and refocused at 8,000V for 10 minutes before equilibration in two equilibration buffers. For the first equilibration (15 minutes), 50 mg of dithiothreitol were added to 200 ml of the stock buffer (2% SDS, 30% glycerol, 6M urea, 50 mM Tris-HCl pH 8.8, and 0.002% bromophenol blue), while for the second equilibration (15 minutes), 125 mg of iodoacetamide were added to 200 ml of stock buffer before use. Following equilibration, the IPG strip was placed directly in contact with the resolving gel. Two protein markers, ColourBurst™ protein markers (20 l; Sigma-Aldrich, St Louis, MO) and Precision Plus Protein™ Unstained Standards (20 l; Bio-Rad, Hercules, CA) were also loaded onto small pieces of filter paper separately before being placed directly in contact with both the left and right sides of the resolving gel next to the IPG strip. Electrophoresis was carried out at 300V constant voltage for 3.5 hours at 4ºC.

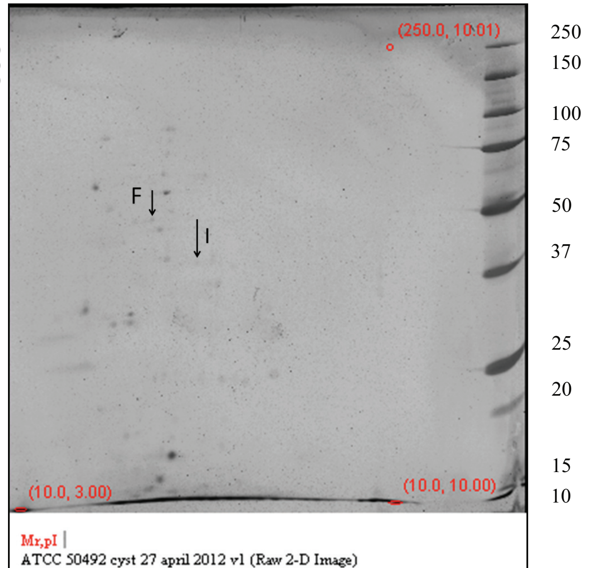
Staining of 2D gels was performed using SilverQuest silver staining kit (Invitrogen, Carlbad, CA). Photographs of gels were taken using GS 800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed using PDQuest 2-D Analysis Software (Bio-Rad Laboratories, Hercules, CA).

## RESULTS

2D-GE performed on protein extracts of trophozoite and cyst stages of *Acanthamoeba* ATCC 50492, IMU 1, IMU4, and IMU5 isolates showed numerous protein spots; some of which appeared to be specific for either trophozoite or cyst while others were found in both stages (shared spots) (Fig 1). The clinical isolate ATCC 50492 had the highest number of protein spots (86 and 45 spots for trophozoite and cyst, respectively), while IMU 1 which was 100% genetically identical to *A. castellanii* appeared to express higher numbers of proteins (69 and 21 spots, respectively) compared to the other 2 environmental isolates, IMU4 and IMU5, which had less genetic identity to *A. castellanii* genotype (Table 1). All the 4 isolates showed more protein spots, including stage-specific proteins, in the trophozoite compared to

A



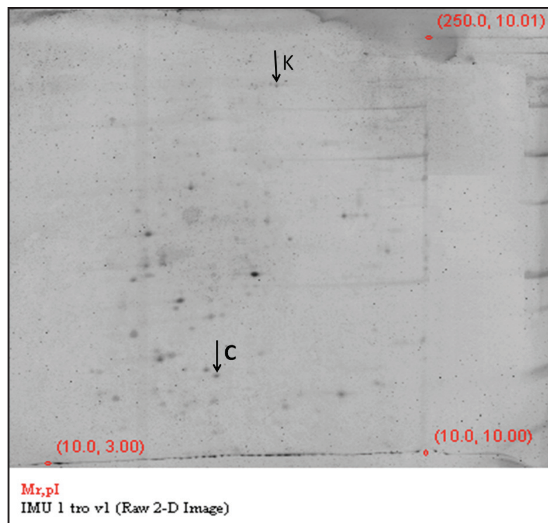

ATCC 50492 trophozoite gel.

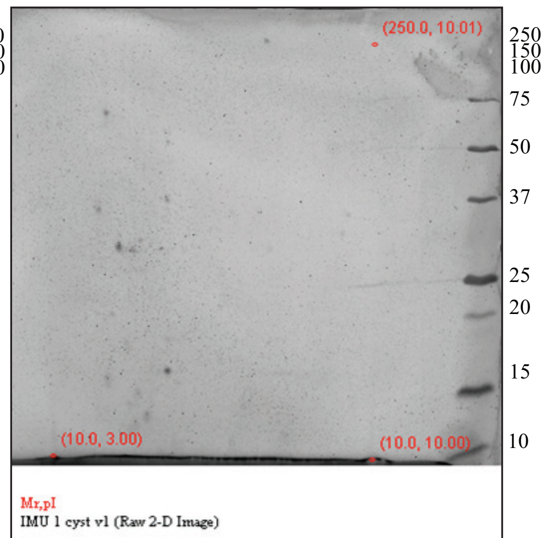
B (15.80 kDa, pI 5.90)
J (97.14 kDa, pI 7.20)

ATCC 50492 cyst gel.

F (47.74 kDa, pI 5.53)
I (39.63 kDa, pI 6.36)

B




IMU1 trophozoite gel.

C (15.61 kDa, pI 6.08)
K(100.07 kDa, pI 7.25)

IMU1 cyst gel.

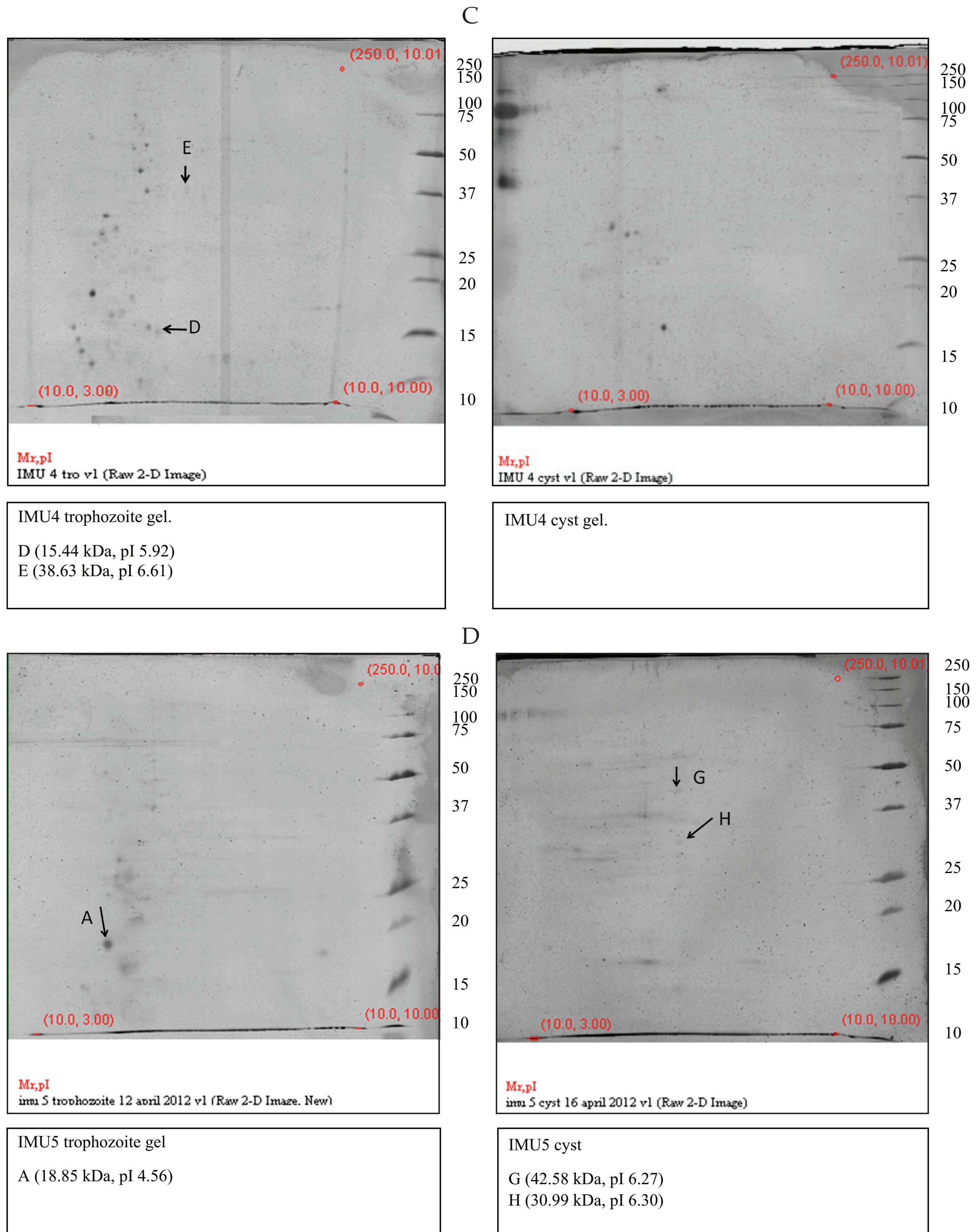



Fig 1–Two-dimensional gels of protein spots of *A. castellanii* trophozoite and cyst strains ATCC 50492 (A), IMU1 (B), IMU4 (C) and IMU5 (D).

Table 2
Stage-specific and shared protein spots with high relative expression in *Acanthamoeba* isolates ATCC50492, IMU 1, IMU4 and IMU5.

| Isolate reference | Spot no. | Relative MW (kDa) | Relative pI | Relative expression in trophozoite | Relative expression in cyst |
|---|---|---|---|---|---|
| ATCC 50492 | 1 | 56.31 | 5.77 | - | 118.8 |
| | 2 | 89.73 | 5.90 | - | 187.5 |
| | 3 | 63.92 | 5.72 | - | 232.1 |
| | 4 | 32.39 | 4.24 | - | 113.8 |
| | 5 | 30.76 | 5.09 | - | 142.2 |
| | 6 | 40.01 | 5.79 | - | 200.4 |
| | 7 | 10.60 | 5.20 | - | 156.1 |
| | 8 | 11.10 | 5.44 | - | 128.5 |
| | 9 | 10.80 | 5.87 | - | 233.0 |
| | 10 | 45.75 | 5.65 | - | 163.8 |
| | 11* | 63.77 | 4.53 | 0.9 | 115.9 |
| | 12* | 26.57 | 5.18 | 41.8 | 130.7 |
| | 13* | 24.80 | 4.95 | 3.2 | 159.7 |
| IMU1 | 1 | 26.44 | 6.78 | 165.5 | - |
| IMU4 | 1* | 15.73 | 5.71 | 19.9 | 164.5 |
| IMU5 | 1 | 18.85 | 4.56 | 242.5 | - |

*Shared spot.

cyst stage. Both ATCC 50492 and IMU1 had 10 shared protein spots while IMU4 and IMU5 had 7 and 2 shared spots, respectively.

Both ATCC 50492 and IMU 1 showed protein spots of higher molecular weights and pI values compared to IMU4 and IMU5. For example, for ATCC 50492, the two highest protein spots are 97.14 kDa, pI 7.20 and 96.18 kDa, pI 6.94 while for IMU 1, they are 100.07 kDa, pI 7.25 and 102.62 kDa, pI 5.46 (Fig 1A and B). For IMU4 and IMU5, the two highest molecular weight and pI spots are 74.59 kDa, pI 5.40 and 59.08, pI 4.40, and 59.27 kDa, pI 5.57 and 49.14 kDa, pI 4.33, respectively (Fig 1C and D).

The stage-specific and shared protein spots differed in expression levels in both trophozoite and cyst stages, with relative expression values ranging from as low as 0.2 to a maximum value of 233 (data not shown). ATCC 50492 had the highest number of cyst-specific protein spots (10) that were highly expressed (relative expression value >100) and 3 other protein spots that were also found in trophozoite (Table 2). Both IMU1 and IMU5 had one highly expressed trophozoite-specific protein spot each and none of them had any highly expressed cyst proteins. None of the trophozoite and cyst-specific protein spots in IMU4 was highly expressed; however there was a shared protein that had a relative expression of 164.5 for the cyst stage. In general, a number of cyst-specific proteins for the 4 isolates had a low to very low relative expression values.

Table 3
Putative trophozoite- and cyst-specific proteins identified by Bouyer *et al* (2009).

| Stage-specific proteins | Calculated MW(kDa)/pI |
|---|---|
| **Trophozoite-specific proteins** | |
| Translationally-controlled tumor protein | 18.7/4.4 |
| Actophorin | 15.5/5.9 |
| Ribosomal protein S12 | 14.41/7.3 |
| Elongation factor 2 | 92.6/6.2 |
| Fructose bisphosphate aldolase | 38.9/7 |
| **Cyst-specific proteins** | |
| Heat shock protein 70 | 69.8/5.5 |
| Enolase | 46.9/5.6 |
| Gelation factor | 92.2/4.6 |
| Subtilisin-like serine protease | 43.7/6.6 |

## DISCUSSION

Analysis of 2D-GE protein profiles of 3 environmental isolates was conducted in order to determine whether differences occurred between the environmental isolates and a clinical isolate, and also whether environmental isolates which had 99% and 100% genetic identity (based on 16 S rDNA sequence) to the *A. castellanii* differed in their protein profiles.

A study by Bouyer *et al* (2009) on *A. castellanii* (ATCC 30234, a yeast culture isolate) utilizing 2D-GE identified a number of proteins of interest in either trophozoite or cyst as listed in Table 3. Not all trophozoite-specific protein spots identified by Bouyer *et al* (2009) were present in the three environmental isolates in this study, probably due to differences in origin of the isolates as ATCC 30234 was derived from a yeast culture isolate.

Leitsch *et al* (2010) reported 3 cyst-specific protein spots (35 kDa, pI 6; 30 kDa, pI 6 and 40 kDa, pI 6), latter identified as actin, during the 8$^{th}$ hour after encystation was induced. In our study, IMU5 (30.99 kDa, pI 6.30) (Fig 1D, spot H) and ATCC 50492 (39.63 kDa, pI 6.36) (Fig 1A, spot I) cysts displayed similar spots.

One of the objectives of our study was to determine differences between a clinical isolate (ATCC 50492) and the 3 environmental isolates of *A. castellanii.* There were differences in the numbers of proteins detected for each strain. Generally there were more proteins expressed in ATCC 50492 and IMU1 compared to IMU4 and IMU5. The level of IMU4 and IMU5 protein expression was lower compared to that of *A. castellanii* strains having 100% genotype identity. There were also differences in the molecular weights of proteins between *A. castellanii* isolates ATCC 50492 and IMU1 and between *A. castellanii* isolates IMU4 and IMU5. The molecular weights of proteins seen in both IMU4 and IMU5 trophozoites were relatively lower than those seen in ATCC 50492 and IMU1. These differences may be due to the fact that genotypes even with slight differences can contribute to variations in protein profiles.

Another interesting observation was that in trophozoites of the 4 isolates studied during 2D-GE, isolates ATCC 50492

and IMU1 demonstrated protein spots with very high molecular weights (> 95 kDa) at relatively higher isoelectric points (> pI 7.00). Such proteins may be involved with the parasite's ability to cause acanthamoebic keratitis. These proteins in ATCC 50492 should be further characterized in order to determine whether they are important in corneal infections. As IMU1 appears to have similar proteins spots that are high in molecular weights and pI values, this isolate needs also to be further studied to determine whether these proteins play a role in pathogenesis.

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