■ ORIGINAL ARTICLE

Comparison of Proteins Secreted into Extracellular Space of Pathogenic and Non-pathogenic *Acanthamoeba castellanii*

Eun-Kyung Moon[1], Hyun-Seo Choi[1], So-Min Park[2], Hyun-Hee Kong[3], Fu-Shi Quan[1,4,*]

[1]Department of Medical Zoology, Kyung Hee University School of Medicine, Seoul 02447, Korea; [2]Department of Biomedical Science, Graduate School, Kyung Hee University, Seoul 02447, Korea; [3]Department of Parasitology, Dong-A University College of Medicine, Busan 49201, Korea; [4]Biomedical Science Institute, Kyung Hee University, Seoul 02447, Korea

Abstract: Pathogenic *Acanthamoeba* spp. cause granulomatous amoebic encephalitis and keratitis. *Acanthamoeba* keratitis (AK) is a rare but serious ocular infection that can result in permanent visual impairment or blindness. However, pathogenic factors of AK remain unclear and treatment for AK is arduous. Expression levels of proteins secreted into extracellular space were compared between *A. castellanii* pathogenic (ACP) and non-pathogenic strains. Two-dimensional polyacrylamide gel electrophoresis revealed 123 differentially expressed proteins, including 34 increased proteins , 7 qualitative increased proteins, 65 decreased proteins, and 17 qualitative decreased proteins in ACP strain. Twenty protein spots with greater than 5-fold increase in ACP strain were analyzed by liquid chromatography triple quadrupole mass spectrometry. These proteins showed similarity each to inosine-uridine preferring nucleoside hydrolase, carboxylesterase, oxygen-dependent choline dehydrogenase, periplasmic-binding protein proteinases and hypothetical proteins. These proteins expressed higher in ACP may provide some information to understand pathogenicity of *Acanthamoeba*.

Key words: *Acanthamoeba castellanii*, secretory product

INTRODUCTION

Acanthamoeba is an opportunistic pathogen capable of causing granulomatous encephalitis and keratitis [1]. *Acanthamoeba* keratitis (AK) is a rare disease. However, the incidence of AK has gradually increased because *Acanthamoeba* has a widespread distribution in the environment while contact lens use is increasing [2,3]. AK is a painful corneal infection that may lead to vision loss or enucleation [4]. The first critical step in the pathogenesis of infection is its adhesion to the surface of host tissues. It is known that mannose binding protein can mediate the adhesion of *Acanthamoeba* to the surface of cornea [5]. Serine and cysteine proteolytic activities of *Acanthamoeba* could play important roles in infections [6]. However, information regarding the mechanism of pathogenic *Acanthamoeba* for adhesion, tissue invasion, and infection is limited.

Proteins secreted into extracellular space as potential targets for treatment and diagnosis of amoebiasis have been reported

[7,8]. During infection, ES proteins released by *Entamoeba histolytica* trophozoites are involved in the invasion into colonic mucosa of the host [8]. A unique cysteine protease in ES products of pathogenic strain of *E. histolytica* has been cloned [7]. Arginine deiminase, ornithine carbamoyl transferase, and enolase have been identified from secreted proteins of *Giardia intestinalis* as immunodominant antigens [9]. During contact with human vaginal epithelial cells, *Trichomonas vaginalis* releases 19 major proteins, including cysteine proteases [10].

In pathogenic strain of *Acanthamoeba*, extracellular serine proteases have been identified as virulence factors [11]. A secretory protease of *A. castellanii* can degrade immunoglobulin A (IgA), IgG, and IgM [12]. Recently, proteomic analysis of secreted proteins by *Acanthamoeba* for non-pathogenic strain ATCC 30010 and clinically pathogenic isolates has been performed [13], revealing 44 secreted proteins, including 10 consensus secretory proteins and 34 strain specific secretory proteins. However, definite virulence factors of pathogenic *Acanthamoeba* have not been found yet. More detailed information about pathogenic strains is needed.

To gain more information on virulence factors of *Acanthamoeba* infection, the objective of this study was to compare the levels of proteins secreted into extracellular space between pathogenic ATCC 30011 strain and non-pathogenic ATCC

30868 strain of *Acanthamoeba*. Differentially expressed proteins were identified, focusing on highly expressed proteins in pathogenic *Acanthamoeba*.

MATERIALS AND METHODS

Cultivation of *Acanthamoeba* and isolation of secreted proteins

Acanthamoeba castellanii non-pathogenic (ACNP) strain (ATCC 30011) and *Acanthamoeba castellanii* pathogenic (ACP) strain (ATCC 30868) were obtained from the American Type Culture Collection, and cultured axenically in PYG medium (20 g/L proteose peptone, 1 g/L yeast extract, 0.1 M glucose, 4 mM MgSO$_4$, 0.4 mM CaCl$_2$, 3.4 mM sodium citrate, 0.05 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 2.5 mM Na$_2$HPO$_4$, and 2.5 mM KH$_2$PO$_4$) at 25°C. Cultured media were harvested after cultivation for 7 days.

Sample preparation and 2-dimensional gel electrophoresis

Sample preparation and 2-dimensional gel electrophoresis (2-DE) were performed as described by Bahk et al. [14]. The cell culture medium (700 ml) was first passed through Amicon Ultra-15 Centrifugal Filter Unit (Merck KGaA, Darmstadt, Germany) at 5,000 g. The harvested samples were suspended in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2.5% (w/v) dithiothreitol (DTT) and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Indiana). The lysates were homogenized and centrifuged at 15,000 g for 20 min and suitably stored at -80°C. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, California, USA). For 2-DE analysis, pH 4-7 immobilized pH gradient (IPG) strips (GE Healthcare Life Sciences, Pittsburg, Pennsylvania) were rehydrated in swelling buffer containing 7 M urea, 2 M thiourea, 2.5% (w/v) DTT, and 4% (w/v) CHAPS. The protein lysates (600 μg) were loaded into the rehydrated IPG strips using an IPGphor III (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania) and the 2-DE separation was performed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After fixation for 1 hr in 40% (v/v) methanol containing 5% (v/v) phosphoric acid, the gels were stained with Colloidal Coomassie Blue G-250 (ProteomeTech, Seoul, Korea). The gels were destained using deionized water and images were acquired with image scanner (Bio-Rad, Hercules, California). Image analysis was carried out using ImageMasterTM 2D Platinum software (Amersham Biosciences, Hercules, California). For comparison of protein spots, more than 25 spots in all

gels were correspondingly landmarked and normalized.

In-gel digestion with trypsin and extracting peptides

Protein spots were excised from SDS-PAGE gels and in-gel digested with trypsin [14]. In brief, the gel pieces were washed for 1 hr at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile. Following dehydration in a centrifugal vacuum concentrator for 10 min, gel pieces were rehydrated in 0.05% trypsin solution (Promega, Madison, Wisconsin). After incubation in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37°C overnight, the tryptic peptides were extracted with 5 μl of 0.5% formic acid containing 50% (v/v) acetonitril for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator. Prior to mass spectrometric analysis, the peptide solution was desalted using a reversed-phase column [15].

Anaylsis on proteins by LC-MS/MS

LC–MS/MS analysis was performed through an agilent 1,100 series nano-LC and LTQ-mass spectrometer (Thermo Electron, San Jose, California). The capillary column used for LC–MS/MS analysis (150 mm×0.075 mm) was obtained from Proxeon (Odense M, Denmark) and slurry packed in house with 5 μm, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, California). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 6% B to 50% B for 22 min, from 50% B to 95% B for 5 min, and from 95% B to 6% B for 13 min. The flow rate was 0.4 μl/min. For tandem mass spectrometry, mass spectra were acquired using data-dependent acquisition with full mass scan (350-1,800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscans on the LTQ. The temperature of the ion transfer tube was controlled at 200°C and the spray was 1.5-2.0 kV. The normalized collision energy was set at 35% for MS/MS. The individual spectra from MS/MS were processed using the SEQUEST software (Thermo Quest, San Jose, California) and the generated peak lists were used to query in house database using the MASCOT program (Matrix Science Ltd., London, UK). We set the modifications of methionine, cysteine, methylation of arginine, and phosphorylation of serine, threonine, and tyrosine for MS analysis and tolerance of peptide mass was 10 ppm. MS/MS ion mass

tolerance was 0.8 Da, allowance of missed cleavage was 1, and charge states (+2 and +3) were taken into account for data analysis. We took only significant hits as defined by MASCOT probability analysis. MS/MS spectra were subjected to batch *de novo* sequencing by PepNovo program, and candidate peptide sequences were used for subsequent MS BLAST searches.

RESULTS

Comparison of proteins secreted into extracellular space of non-pathogenic and pathogenic strain of *A. castellanii*

In 2-DE gels, excretory-secretory (ES) products harvested from culture media after cultivation for 7 days showed more protein spots than those after 3 days or 5 days of culture. For 2-DE analysis, broad-range pH gradients (pH 3-10) and narrow range pH gradient (pH 4-7) strips were used for ES proteins of *Acanthamoeba*. Protein spots were determined to be weakly acidic as they were concentrated along the center of the broad-range pH gradient (pH 3-10) strip. Narrow range pH

gradient (pH 4-7) strip provided enhanced resolution as these protein spots were evenly distributed throughout the strip. ES proteins of ACNP and ACP strians were evaluated by pH 4-7 strip and 10% SDS-PAGE. Compared to the ACNP strain, the ACP strain showed 123 differentially expressed proteins, where 34 proteins increased (IN), 7 proteins qualitatively increased (QI), 65 proteins decreased (DE), and 17 proteins qualitatively decreased (QD). IN refers to proteins that are present in ACNP but significantly increased in ACP. QI refers to proteins that are absent in ACNP but expressed in ACP. DE refers to proteins that are expressed in ACNP, but decreased in ACP. Finally, QD refers to proteins that are not expressed in ACP but expressed in ACNP.

Proteins up-regulated in the pathogenic *A. castellanii*

ACP strain revealed 34 INs (Fig. 1A, B) and 7 QIs (Fig. 1C, D). Supplementary Table S1 shows value of image analysis of increased proteins (from IN01 to IN34) in Fig. 1A and B. Supplementary Table S2 shows value of image analysis for qualitatively

Fig. 1. Two-dimensional gel electrophoresis of up-regulated proteins in *A. castellanii*. Comparison of ES proteins between ACNP strain (A, C) and ACP strain (B, D) revealed 34 proteins with increased expression (B) and 7 proteins demonstrating qualitative increase (D) in the ACP strain. Numbers in Fig. 1 represents spot identification numbers listed in Supplementary Tables S1 and S2.
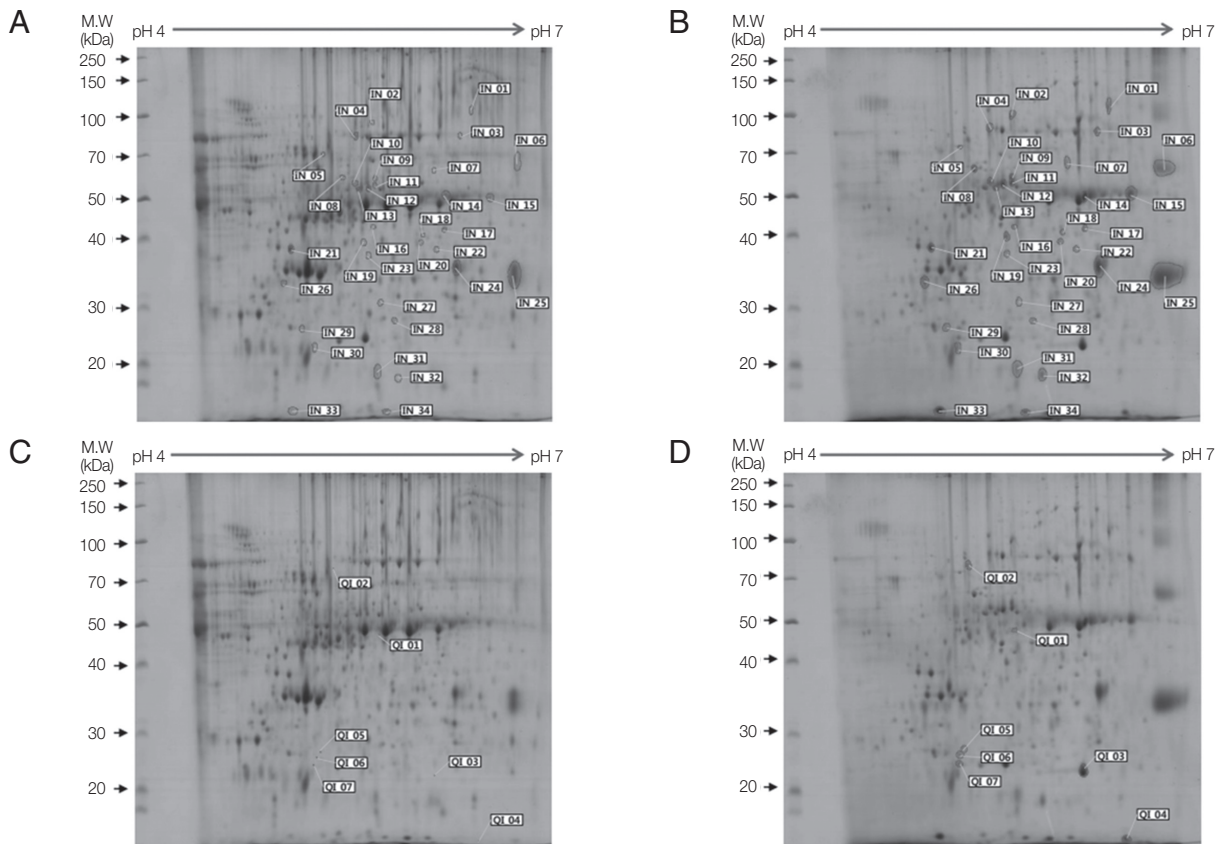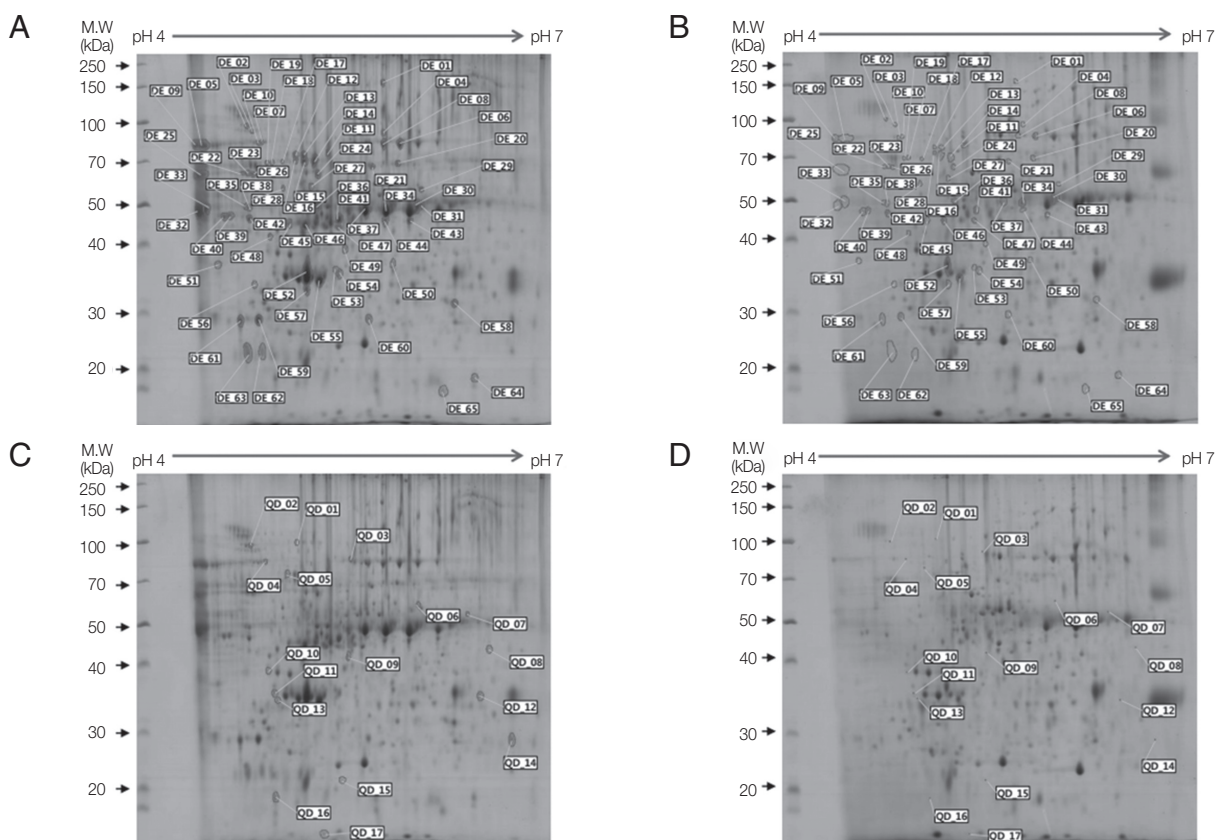
Fig. 2. Two-dimensional gel electrophoresis of down-regulated proteins in *A. castellanii*. Comparision of ES proteins between ACNP strain (A, C) and ACP strain (B, D) showed 65 decreased proteins (B) and 17 qualitatively decreased proteins (D) in the ACP strain. Numbers in Fig. 2 represents spot identification numbers listed in Supplementary Tables S3 and S4.

increased proteins (from QI01 to QI07) in Fig. 1C and D.

Proteins down-regulated in the pathogenic *A. castellanii*

There were 65 decreased proteins (Fig. 2A, B) and 17 qualitatively decreased proteins (Fig. 2C, D) in the ACP strain compared to ES proteins in the ACNP strain. Supplementary Table S3 shows value of the image analysis of decreased proteins (from DE01 to DE65) in Fig. 2A and B. Supplementary Table S4 shows value of image analysis for qualitatively decreased proteins (from QD01 to QD17) in Fig. 2C and D.

Proteins highly expressed in pathogenic *A. castellanii*

Among 41 up-regulated protein spots, 13 proteins with more than 5-fold higher expression and 7 qualitative increase proteins were selected for protein identification. To identify these highly expressed ES proteins in ACP strain, 20 protein spots were analyzed by LC–MS/MS. Results of the mass spectrometer and database search for 20 highly expressed protein spots are summarized in Table 1. Nine out of 20 protein spots

were related to *Acanthamoeba* (Table 1). Hypothetical protein ACA_279470 (IN02, IN11, and QI02) contained a periplasmic-binding protein domain. One protein spot (IN15) showed similarity with inosine uridine preferring nucleoside hydrolase. Another spot (IN01) showed similarity with carboxylesterase of *A. castellanii*. Protein spots of IN08 and QI06 were similar to oxygen-dependent choline dehydrogenase of *Rhizoctonia solani*. Unfortunately, IN06, IN18, and IN32 were hypothetical proteins.

DISCUSSION

To search for pathogenic factors of *Acanthamoeba*, ES proteins of *A. castellanii* non-pathogenic (ACNP) strain and *A. castellanii* pathogenic (ACP) strain were compared by 2-dimensional polyacrylamide gel electrophoresis. Observation of distinct protein spots revealed 123 differentially expressed proteins, including 41 up-regulated proteins and 82 down-regulated proteins in ACP strain compared to those in the ACNP strain.

Table 1. Proteins up-regulated 5-times or more in ACP strain

Group ID	Description	Score	Mass (kDa)
IN01	carboxylesterase superfamily protein [*Acanthamoeba castellanii* Neff]	65	57.9
IN02	hypothetical protein ACA1_279470 [*Acanthamoeba castellanii* Neff]	82	63.1
IN03	ALB protein [*Bos taurus*]	81	71.2
IN06	hypothetical protein ACA1_362140 [*Acanthamoeba castellanii* Neff]	100	25.3
IN07	ALB protein [*Bos taurus*]	65	71.2
IN08	oxygen-dependent choline dehydrogenase [*Rhizoctonia solani*]	45	70.0
IN11	hypothetical protein ACA1_279470 [*Acanthamoeba castellanii* Neff]	154	63.1
IN15	inosine uridine preferring nucleoside hydrolase family protein [*Acanthamoeba castellanii* Neff]	74	38.0
IN16	hypothetical protein BBAD15_g11018 [*Beauveria bassiana* D1-5]	17	60.8
IN18	hypothetical protein ACA1_052470 [*Acanthamoeba castellanii* Neff]	54	38.7
IN26	adaptor protein complex AP-3 delta subunit [*Sphaerulina musiva* SO2202]	19	116.4
IN32	hypothetical protein ACA1_132680 [*Acanthamoeba castellanii* Neff]	50	19.6
IN33	hypothetical protein I79_015947 [*Cricetulus griseus*]	26	14.3
QI01	prokumamolisin, activation domain containing protein [*Acanthamoeba castellanii* Neff]	50	63.6
QI02	hypothetical protein ACA1_279470 [*Acanthamoeba castellanii* Neff]	64	63.1
QI03	RIKEN cDNA 4732456N10, partial [*Mus musculus*]	108	59.3
QI04	hypothetical protein PB105628.00.0, partial [*Plasmodium berghei*]	26	4.9
QI05	hypothetical protein BCV71DRAFT_164154, partial [*Rhizopus microsporus*]	37	17.1
QI06	oxygen-dependent choline dehydrogenase [*Rhizoctonia solani*]	45	66.9
QI07	odorant-binding protein 19c [*Drosophila pseudoobscura* pseudoobscura]	21	19.9

Twenty protein spots highly expressed in ACP strain were isolated from gels for identification (Table 1). All 20 spots were identified, nine were related to *Acanthamoeba* database.

Among these protein spots, 3 spots (IN02, IN11, and QI02) were identified as hypothetical protein of *Acanthamoeba* (Table 1). The aforementioned IN02, IN11, and QI02 included periplasmic binding protein region. Periplasmic binding proteins (PBPs) are nonenzymatic receptors that bacteria can use to sense small molecules and transport them into the cytoplasm [16]. Most PBPs participate in the transport of solute molecules into the cytoplasm via ATP-binding cassette transporters [17]. Most Gram-negative bacteria are thought to employ periplasmic-binding-protein-dependent transport. Periplasmic chaperone in *Camphylobacter jejuni* is involved in its invasion to cultured human epithelial cells [18].

One protein spot highly expressed in the ACP strain (IN01) showed similarity with carboxylesterase superfamily protein of *A. castellanii* Neff (Table 1). Carboxylesterases are widely distributed in nature. Carboxylates are conjugated by other enzymes to increase solubility and eventually excreted. A cell wall-associated carboxylesterase has been reported to be required for virulence of *Mycobacterium tuberculosis* [19].

Another protein spot highly expressed in the ACP strain (IN15) showed similarity with inosine-uridine preferring nucleoside hydrolase of *Acanthamoeba* (Table 1). Inosine-uridine preferring nucleoside hydrolase is an enzyme that catalyses the hydrolysis of commonly occuring purine and pyrimidine nucleosides into ribose and associated base. This enzyme is important for parasitic organisms known to be deficient in de novo synthesis of purines to salvage host purine nucleosides. *Plasmodium falciparum* is a purine auxotroph. It salvages host cell purines for synthesis of cofactors and nucleic acids [20].

Two protein spots in ACP strain (IN08 and QI06) showed similarity with oxygen-dependent choline dehydrogenase of *Rhizoctonia solani* (Table 1). Choline dehydrogenase is an enzyme catalyzing the dehydrogenation of choline to betaine aldehyde. Choline dehydrogenase plays a dual role (choline conversion and mitophagy) in the membrane of mitochondria [21].

To date, biochemical and kinetic properties of proteins highly expressed in ACP have been poorly characterized. We cloned 6 cDNAs, which encodes highly expressed proteins in the ACP (periplasmic binding protein, carboxyl esterase, inosine uridine preferring nucleoside hydrolase, chorismate mutase, adenylyl cyclase associated protein, and prokumamolisin). We plan on identifying characteristics associated with pathotoxicity for these proteins, and develop rapid and sensitive diagnostic methods.

Characterization of highly expressed secretory proteins in pathogenic *Acanthamoeba* will provide better understanding about its pathogenesis. It might lead to early diagnosis and

treatment for *Acanthamoeba* infections.

ACKNOWLEDGMENT

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

1. Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev 2003; 16: 273-307.

2. Jones DB, Visvesvara GS, Robinson NM. *Acanthamoeba polyphaga* keratitis and *Acenthamoeba uveitis* associated with fatal meningoencephalitis. Trans Ophthalmol Soc UK 1975; 95: 221-232.

3. Verani JR, Lorick SA, Yoder JS, Beach MJ, Braden CR, Roberts JM, Conover CS, Chen S, McConnell KA, Chang DC, Park BJ, Jones DB, Visvesvara GS, Roy SL. National outbreak of *Acanthamoeba* keratitis associated with use of a contact lens solution, United States. Emerg Infect Dis 2009; 15: 1236-1242.

4. Awwad ST, Petroll WM, McCulley JP, Cavanagh HD. Updates in *Acanthamoeba* keratitis. Eye Contact Lens 2007; 33: 1-8.

5. Garate M, Marchant J, Cubillos I, Cao Z, Khan NA, Panjwani N. In vitro pathogenicity of *Acanthamoeba* is associated with the expression of the mannose-binding protein. Invest Ophthalmol Vis Sci 2006; 47: 1056-1062.

6. Serrano-Luna Jde J, Cervantes-Sandoval I, Calderón J, Navarro-García F, Tsutsumi V, Shibayama M. Protease activities of *Acanthamoeba polyphaga* and *Acanthamoeba castellanii*. Can J Microbiol 2006; 52: 16-23.

7. Reed S, Bouvier J, Pollack AS, Engel JC, Brown M, Hirata K, Que X, Eakin A, Hagblom P, Gillin F. Cloning of a virulence factor of *Entamoeba histolytica*. Pathogenic strains possess a unique cysteine proteinase gene. J Clin Invest 1993; 91: 1532-1540.

8. Moncada D, Keller K, Chadee K. *Entamoeba histolytica*-secreted products degrade colonic mucin oligosaccharides. Infect Immun 2005; 73: 3790-3793.

9. Ringqvist E, Palm JE, Skarin H, Hehl AB, Weiland M, Davids BJ, Reiner DS, Griffiths WJ, Eckmann L, Gillin FD, Svärd SG. Release of metabolic enzymes by *Giardia* in response to interaction with

10. Kucknoor AS, Mundodi V, Alderete JF. The proteins secreted by *Trichomonas vaginalis* and vaginal epithelial cell response to secreted and episomally expressed AP65. Cell Microbiol 2007; 9: 2586-2597.

11. Lorenzo-Morales J, Ortega-Rivas A, Foronda P, Abreu-Acosta N, Ballart D, Martínez E, Valladares B. RNA interference (RNAi) for the silencing of extracellular serine proteases genes in *Acanthamoeba*: molecular analysis and effect on pathogenecity. Mol Biochem Parasitol 2005; 144: 10-15.

12. Na BK, Cho JH, Song CY, Kim TS. Degradation of immunoglobulins, protease inhibitors and interleukin-1 by a secretory proteinase of *Acanthamoeba castellanii*. Korean J Parasitol 2002; 40: 93-99.

13. Huang JM, Lin WC, Li SC, Shih MH, Chan WC, Shin JW, Huang FC. Comparative proteomic analysis of extracellular secreted proteins expressed by two pathogenic *Acanthamoeba castellanii* clinical isolates and a non-pathogenic ATCC strain. Exp Parasitol 2016; 166: 60-67.

14. Bahk YY, Kim SA, Kim JS, Euh HJ, Bai GH, Cho SN, Kim YS. Antigens secreted from *Mycobacterium tuberculosis*: identification by proteomics approach and test for diagnostic marker. Proteomics 2004; 4: 3299-3307.

15. Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. Sample purification and preparation technique based on nanoscale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. J Mass Spectrom 1999; 34: 105-116.

16. Quiocho FA. Atomic structures of periplasmic binding proteins and the high-affinity active transport systems in bacteria. Philos Trans R Soc Lond B Biol Sci 1990; 326: 341-351.

17. Tam R, Saier MH Jr. Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. Microbiol Rev 1993; 57: 320-346.

18. Kervella M, Pagès JM, Pei Z, Grollier G, Blaser MJ, Fauchère JL. Isolation and characterization of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membranes. Infect Immun 1993; 61: 3440-3448.

19. Lun S, Bishai WR. Characterization of a novel cell wall-anchored protein with carboxylesterase activity required for virulence in *Mycobacterium tuberculosis*. J Biol Chem 2007; 282: 18348-18356.

20. Hyde JE. Targeting purine and pyrimidine metabolism in human apicomplexan parasites. Curr Drug Targets 2007; 8: 31-47.

21. Park S, Choi SG, Yoo SM, Son JH, Jung YK. Choline dehydrogenase interacts with SQSTM1/p62 to recruit LC3 and stimulate mitophagy. Autophagy 2014; 10: 1906-1920.

intestinal epithelial cells. Mol Biochem Parasitol 2008; 159: 85-91.

Supplementary Table S1. Image analysis of proteins increased (IN) in ACP strain

Group ID	ACNP strain	ACP strain	Ratio (100%)	Separability
IN01	0.0364	0.2743	7.5423	0.2379
N02	0.0171	0.2544	14.8874	0.2373
IN03	0.0215	0.1514	7.0487	0.1299
IN04	0.1246	0.2768	2.2209	0.1522
IN05	0.0209	0.0513	2.4492	0.0303
IN06	0.1517	1.5261	10.0611	1.3744
IN07	0.0130	0.2060	15.9002	0.1931
IN08	0.0317	0.2632	8.3107	0.2316
IN09	0.0603	0.2348	3.8902	0.1744
IN10	0.0772	0.2147	2.7820	0.1375
IN11	0.0344	0.2429	7.0647	0.2085
IN12	0.1010	0.4331	4.2878	0.3321
IN13	0.0546	0.1809	3.3158	0.1264
IN14	0.2255	0.7624	3.3817	0.5370
IN15	0.1458	0.9388	6.4376	0.7930
IN16	0.0115	0.0946	8.2033	0.0830
IN17	0.0465	0.1101	2.3691	0.0636
IN18	0.0108	0.0673	6.2427	0.0566
IN19	0.0414	0.1978	4.7830	0.1565
IN20	0.0343	0.0766	2.2300	0.0422
IN21	0.2251	0.4597	2.0418	0.2345
IN22	0.0285	0.0754	2.6473	0.0469
IN23	0.0221	0.0697	3.1604	0.0477
IN24	0.7647	1.6932	2.2142	0.9285
IN25	1.7750	5.8906	3.3187	4.1156
IN26	0.0576	0.5182	8.9997	0.4606
IN27	0.0370	0.1003	2.7140	0.0633
IN28	0.0428	0.1071	2.5050	0.0644
IN29	0.0485	0.1320	2.7209	0.0835
IN30	0.0776	0.3161	4.0716	0.2385
IN31	0.2405	0.5853	2.4336	0.3448
IN32	0.0341	0.4789	14.0626	0.4448
IN33	0.1110	0.7666	6.9095	0.6557
IN34	0.1171	0.2509	2.1419	0.1338

Supplementary Table S2. Image analysis of proteins qualitatively increased (QI) in ACP strain

Group ID	ACNP strain	ACP strain	Ratio (100%)	Separability
QI01	0.0003	0.0449	129.71	0.0445
QI02	0.0002	0.2025	1,104.19	0.2023
QI03	0.0001	1.1205	8,910.35	1.1203
QI04	0.0001	0.3703	2,718.26	0.3702
QI05	0.0001	0.1025	1,087.23	0.1024
QI06	0.0001	0.1279	1,061.66	0.1278
QI07	0.0002	0.1433	854.394	0.1431

Supplementary Table S3. Image analysis of proteins decreased (DE) in ACP strain

Group ID	ACNP strain	ACP strain	Ratio (100%)	Separability
DE01	0.0266	0.0096	2.7801	0.0171
DE02	0.0449	0.0087	5.1403	0.0361
DE03	0.0687	0.0099	6.9096	0.0587
DE04	0.1264	0.0239	5.2795	0.1025
DE05	0.0646	0.0163	3.9729	0.0483
DE06	0.1559	0.0214	7.2788	0.1345
DE07	0.0464	0.0117	3.9778	0.0347
DE08	0.0813	0.0360	2.2585	0.0453
DE09	0.2109	0.0265	7.9713	0.1844
DE10	0.2818	0.0088	32.0313	0.2730
DE11	0.0381	0.0099	3.8538	0.0282
DE12	0.0634	0.0129	4.9307	0.0506
DE13	0.1147	0.0324	3.5354	0.0823
DE14	0.0927	0.0203	4.5718	0.0724
DE15	0.0881	0.0300	2.9408	0.0581
DE16	0.0868	0.0305	2.8492	0.0563
DE17	0.5084	0.0243	20.8984	0.4841
DE18	0.2876	0.0267	10.7850	0.2609
DE19	0.1208	0.0176	6.8627	0.1032
DE20	0.1525	0.0393	3.8795	0.1132
DE21	0.0633	0.0248	2.5565	0.0386
DE22	0.2947	0.0295	9.9861	0.2652
DE23	0.0266	0.0096	2.7801	0.0171
DE24	0.0449	0.0087	5.1403	0.0361
DE25	0.0687	0.0099	6.9096	0.0587
DE26	0.1264	0.0239	5.2795	0.1025
DE27	0.0646	0.0163	3.9729	0.0483
DE28	0.1559	0.0214	7.2788	0.1345
DE29	0.0464	0.0117	3.9778	0.0347
DE30	0.0813	0.0360	2.2585	0.0453
DE31	0.2109	0.0265	7.9713	0.1844
DE32	0.2818	0.0088	32.0313	0.2730
DE33	0.0381	0.0099	3.8538	0.0282
DE34	0.0634	0.0129	4.9307	0.0506
DE35	0.1147	0.0324	3.5354	0.0823
DE36	0.0927	0.0203	4.5718	0.0724
DE37	0.0881	0.0300	2.9408	0.0581
DE38	0.0868	0.0305	2.8492	0.0563
DE39	0.5084	0.0243	20.8984	0.4841
DE40	0.2876	0.0267	10.7850	0.2609
DE41	0.1208	0.0176	6.8627	0.1032
DE42	0.1525	0.0393	3.8795	0.1132
DE43	0.0633	0.0248	2.5565	0.0386
DE44	0.2947	0.0295	9.9861	0.2652
DE45	0.0266	0.0096	2.7801	0.0171
DE46	0.0449	0.0087	5.1403	0.0361
DE47	0.0687	0.0099	6.9096	0.0587
DE48	0.1264	0.0239	5.2795	0.1025
DE49	0.0646	0.0163	3.9729	0.0483
DE50	0.1559	0.0214	7.2788	0.1345
DE51	0.0464	0.0117	3.9778	0.0347
DE52	0.0813	0.0360	2.2585	0.0453
DE53	0.2109	0.0265	7.9713	0.1844
DE54	0.2818	0.0088	32.0313	0.2730
DE55	0.0381	0.0099	3.8538	0.0282
DE56	0.0634	0.0129	4.9307	0.0506
DE57	0.1147	0.0324	3.5354	0.0823
DE58	0.0927	0.0203	4.5718	0.0724
DE59	0.0881	0.0300	2.9408	0.0581
DE60	0.0868	0.0305	2.8492	0.0563
DE61	0.5084	0.0243	20.8984	0.4841
DE62	0.2876	0.0267	10.7850	0.2609
DE63	0.1208	0.0176	6.8627	0.1032
DE64	0.1525	0.0393	3.8795	0.1132
DE65	0.0633	0.0248	2.5565	0.0386

Supplementary Table S4. Image analysis of proteins qualitatively decreased (QD) in ACP strain

Group ID	ACNP strain	ACP strain	Ratio (100%)	Separability
QD01	0.0259	0.0001	289.185	0.0258
QD02	0.0465	0.0000	3,379.990	0.0465
QD03	0.0400	0.0001	342.373	0.0399
QD04	0.0366	0.0001	280.289	0.0365
QD05	0.0636	0.0000	1,540.050	0.0635
QD06	0.0550	0.0001	380.499	0.0548
QD07	0.0344	0.0001	333.534	0.0343
QD08	0.0639	0.0001	1,032.480	0.0639
QD09	0.0696	0.0002	460.103	0.0695
QD10	0.0794	0.0001	549.457	0.0792
QD11	0.1123	0.0002	544.176	0.1121
QD12	0.1295	0.0002	723.960	0.1293
QD13	0.0667	0.0001	1,212.760	0.0667
QD14	0.1780	0.0002	995.423	0.1778
QD15	0.0400	0.0001	387.402	0.0399
QD16	0.1467	0.0001	2,665.750	0.1466
QD17	0.0973	0.0002	589.384	0.0971