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Application of proteomics for comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites

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Abstract

Protein profiles of two isolates of *Neospora caninum* (KBA-2 and JPA1) and *Toxoplasma gondii* RH strain were investigated by proteomic approach. Approximately, 78% of protein spots on two-dimensional gel electrophoresis (2-DE) profiles and 80% of antigen spots on 2-DE immunoblotting profiles were exhibited to share the same p*I* and M_r between KBA-2 and JPA1 of *N. caninum*. On the other hand, a total of 30 antigen spots of *T. gondii* were recognized on 2-DE immunoblotting profile using rabbit antiserum against *N. caninum* KBA-2. A number of homologue proteins, such as heat shock protein 70, tubulin α- and β-chain, putative protein disulfide isomerase, actin, enolase and 14-3-3 protein homologue are believed as the conserved proteins in both *N. caninum* and *T. gondii*. On the contrary, NcSUB1, NcGRA2 and NCDG1 (NcGRA7) might be the species-specific proteins for *N. caninum* tachyzoites. The present study showed that the high degree of similarity between *N. caninum* isolates (KBA-2 and JPA1), whereas large differences between *N. caninum* and *T. gondii* were noticed by proteome comparisons.

Keywords: *Neospora caninum*; *Toxoplasma gondii*; Proteome analysis; Two-dimensional gel electrophoresis; Mass spectrometry; Antigen spots

1. Introduction

Neospora caninum and *Toxoplasma gondii* are obligate, cyst-forming intracellular apicomplexan parasites. *N. caninum* was misdiagnosed as *T. gondii* due to their morphological and biological similarities until at the mid-1980s. Recently, *N. caninum* was distinguished from *T. gondii* based on ultrastructural features of the tachyzoites, bradyzoites and tissue cysts. The differential morphological features include the number, appearance and location of rhoptries, micronemes, dense granules and micropores. Especially, morphology of tissue cysts is quite different from each other [1–4]. In molecular phylogenetic analysis based on small subunit ribosomal RNA (18S rRNA) sequence, *N. caninum* was placed as a sister group to *T. gondii* in the phylum Apicomplexa [5]. Comparison of nuclear small subunit ribosomal RNA [6], internal transcribed spacer 1 [7] and large subunit ribosomal RNA gene [8] also revealed that the *N. caninum* and *T. gondii* are closely related, but distinct species.

Analysis of protein profiles would be valuable for obtaining a comprehensive understanding on several aspects

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of biological proceedings including development, evolution and pathogenicity of these organisms. Proteomics involves the systematic analysis of gene expression at protein level [9]. The two-dimensional gel electrophoresis (2-DE) with powerful image analysis software and biological mass spectrometry in combination with database searching made it possible to analyze complex protein mixtures extracted from cells, tissues, or other biological samples [10,11]. These proteomic methods have been proved successfully for characterizing the proteome of *T. gondii* [12] and 2-DE combined with immunoblotting assay enables characterizing the antigen profiles of *T. gondii* using specific antibodies [13,14]. The comparison of 2-DE antigen profiles between *N. caninum* and *T. gondii* has been conducted by using specific antisera [15], however, they could not identify the antigen spots that showing different 2-DE profiles between them.

We previously established 2-DE map of *N. caninum* tachyzoites including conserved proteins between *N. caninum* and *T. gondii* [16]. On the basis of previous study, the protein and antigen profiles expressed in tachyzoites of two isolates of *N. caninum* between Korea and Japan (KBA-2 and JPA1), in addition, between *N. caninum* and *T. gondii* tachyzoites were compared by proteomic approach.

2. Materials and methods

2.1. Chemicals and reagents

Percoll™, urea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), immobilized pH gradient (IPG) strip (Immobiline DryStrip, pH 4–7 liner, 13 cm), IPG-buffer (pH 3–10 and 4–7) and ECL™ detection reagent were obtained from Amersham Bioscience (Uppsala, Sweden). Aceton, acetonitril, 2-propanol, trifluoroacetic acid (TFA) and Coomassie brilliant blue G-250 were purchased from Merck (Darmstadt, Germany). Unless stated otherwise, all reagents and chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Maintenance and purification of parasites

N. caninum KBA-2 [17] and JPA1 [18] strain, and *T. gondii* (RH strain) tachyzoites were maintained in Vero cell monolayer (CRL6318, ATCC, Rockville, USA) according to Kim et al. [17].

N. caninum and *T. gondii* tachyzoites were harvested by scraping infected Vero cell monolayers into growth medium. The suspension was loaded on 30, 50 and 80% (v/v) osmotic Percoll™ gradient and centrifuged at 2000 × *g* for 30 min. A viable tachyzoites band formed between 50 and 80% on the osmotic Percoll™ gradient were collected and washed three times with phosphate buffered saline (PBS, pH 7.4). Approximately 1×10^8 tachyzoites (counted using a hemocytometer) were purified from infected cells and stored at −70 °C until required.

2.3. Production of rabbit-antiserum against N. caninum KBA-2 tachyzoites

Anti-*N. caninum* polyclonal antibodies were raised by immunizing a New Zealand White rabbit which was serologically negative by indirect fluorescence antibody test (IFAT) using antigen slide of *N. caninum* and *T. gondii* tachyzoites [18]. The rabbit was immunized subcutaneously with 1×10^7 live tachyzoites (KBA-2) mixed with Freund's adjuvants three times at 2-week intervals. Final booster was done 1 week after last immunization. Antiserum was collected by heart puncture 1 week after final injection. IFAT titer of antiserum was 1:6400.

2.4. Two-dimensional gel electrophoresis

Purified tachyzoites were dissolved in 40 mM Tris-base, disrupted three times by freeze–thaw cycle in liquid nitrogen, and then sonicated (XL-2020, Misonix Inc., Farmingdale, USA) at 5.5 W for 2 min (5 s pulse/10 s rest) on ice slurry. The disrupted tachyzoites were mixed with lysis buffer composed of 7 M urea, 2 M thiourea, 40 mM Tris-base, 4% (w/v) CHAPS, 1% (w/v) DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.5% (v/v) IPG buffer (pH 3–10). The samples were kept for 1 h in ice slurry and then centrifuged at 16,000 × *g* for 30 min at 4 °C. Protein concentrations of the resulting supernatants were determined by the Bradford method [19] using bovine serum albumin as a standard.

Isoelectric focusing (IEF) was performed using an IPGphor™ system [11]. The tachyzoite samples mixed with rehydration buffer containing 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.4% (w/v) DTT, 0.5% IPG buffer and 0.002% (w/v) bromophenol blue, were loaded on IPG strips (pH 4–7) by in gel rehydration and focused for a total of 86.1 kV h. The IPG strips were then subjected to 10% SDS-polyacrylamide gels (160 mm × 160 mm × 1 mm). The gels were visualized with silver nitrate [20] or colloidal Coomassie blue G-250 [21].

2.5. Immunoblotting and image analysis

Gels after 2-DE separation were transferred to a polyvinylidene fluoride membrane (Immobilon™-P, Millipore, Bedford, USA). The blotted membranes were blocked with TBS-T buffer [20 mM Tris–HCl, 500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4] containing 5% (w/v) skim milk overnight at 4 °C. The membranes were incubated with rabbit antiserum against *N. caninum* KBA-2 diluted at 1:200 for 2 h and subsequently with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, USA) in dilution of 1:2000 for 1 h. Immuno-reactive spots were visualized by ECL™ detection kit.

Stained and immunoblotted spots were digitalized using an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Mortsel, Belgium) and the acquired images were analyzed using