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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

Phoretix™ 2D software (Nonlinear Dynamics, Newcastle, UK). The gel images were calibrated for M_r and p*I* using 2D SDS-PAGE Standards (Bio-Rad, Hercules, USA).

2.6. Protein identification by mass spectrometry

In gel digestion was done essentially as previously described [22]. Protein spots of interest were excised from stained gels. The gel pieces were washed with 50% acetonitrile, dried, after and rehydrated in 10 mM DTT/100 mM NH_4HCO_3 at room temperature (RT). The gel pieces were further incubated in 55 mM iodoacetamide/100 mM NH_4HCO_3 at RT in the dark, washed with 50 mM NH_4HCO_3 and dried again. They were rehydrated in digestion buffer composed of 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 ng/μl of sequence-grade modified porcine trypsin (Promega, Madison, USA) and incubated on ice. After being remove the excess liquid, they were subject to overnight digestion. The supernatants recovered extracted in 1:1 (v/v) mixture of 5% formic acid:acetonitrile, pooled and dried in a vacuum centrifuge.

The tryptic digests were redissolved in a 2 μl solution containing distilled water, acetonitrile and trifluoroacetic acid (93:5:2). The target preparation for MALDI-TOF mass spectrometry was followed by a solution-phase nitrocellulose method [23]. α-Cyano-4-hydroxycinamic acid (40 mg/ml) and nitrocellulose (20 mg/ml) were separately prepared in acetone and mixed with isopropanol. The sample peptide prepared by trypsin digestion was mixed with internal standards of des-Arg-Bradykinin (monoisotopic mass, 904.4681) and angiotensin I (1296.6853). The solution (1 μl) was spotted onto a circle of target plate and dried. The sample spots on the target were analyzed using a Voyager-DE STR MALDI-TOF MS (PerSeptive Biosystems, Franingham, USA). Mass spectra were acquired as ion signals generated by irradiating the target with 128 laser pulses. Monoisotopic peptide masses were selected in the mass range of 800–2500 Da. Proteins were identified by PMF using MS-FIT (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) and the National Center for Biotechnology Information (NCBI) protein sequence database. The mass tolerance was ±50 ppm and one missed cleavage site was allowed. Carbamidomethylation of cysteines and oxidation of methionine were considered during the search.

3. Results

3.1. Protein and immunoblot profiles of N. caninum and T. gondii

Approximately 516 and 573 protein spots were observed from silver staining of 2-DE of KBA-2 and JPA1, respectively. Of these, 78% (403/516 spots) of protein spots were found to have same p*I* and M_r values compared to each other (Fig. 1A and C), and 94 and 91 antigen spots were only detected for KBA-2 and JPA1, respectively, by immunoblottings probed with rabbit-antiserum against *N. caninum* KBA-2 (Fig. 1B and D). Among these immunoreactive spots, 80% (73/91 spots) of JPA1 strain was recognized to be same spot positions with silver stained protein spots in terms of p*I* and M_r. On the other hand, a total of 419 protein spots were observed from *T. gondii* RH strain stained with silver nitrate, of which 30 antigen spots were recognized by immunoblotting against rabbit antiserum against *N. caninum* KBA-2 (Fig. 1E and F).

3.2. Protein identification from 2-DE maps of N. caninum and T. gondii

On the basis of 2-DE map of *N. caninum* previously established, some proteins were also identified from 2-DE map of *T. gondii* [16]. In this study, three protein spots such as subtilisin-like serine protease (NcSUB1, spot 21), lactate dehydrogenase (spot 43) and serine–threonine phosphatase 2C (spot 45) were newly identified from 2-DE map of *N. caninum*. Eleven protein spots corresponding to nine different proteins were also identified from 2-DE map of *T. gondii* using PMF analysis. The newly identified protein spots were summarized in Table 1.

3.3. Comparison of N. caninum and T. gondii protein spots

Protein spots of *N. caninum* (KBA-2 and JPA1) and *T. gondii* were further analyzed by dividing the 2-DE maps of each strain into seven different parts. Fig. 2 shows a protein profile of the part 1, in which heat shock protein 70 (HSP 70; N-2 and T-2), tubulin β-chain (N-7 and T-5), putative protein disulfide isomerase (N-9 and T-7) and actin (N-10 and T-8) were found to locate in similar places of *N. caninum* and *T. gondii* at the range of between pH 4.7 and 5.1, with the molecular weights ranged from 41 to 76 kDa. Although T-6 of *T. gondii* was not identified, it located similar positions with tubulin α-chain (N-8) of *N. caninum*.

As seen in Fig. 3 (protein profile of the part 2), some protein spots showed identical patterns between *N. caninum* and *T. gondii*. N-1, N-3 and N-5 of *N. caninum*, which are equivalent to T-1, T-3 and T-4 of *T. gondii*, appeared at similar locations at pH ranges between 4.8 and 5.4 and molecular weight between 52 and 76 kDa. These spots were not identified, but T-1, T-3 and T-4 of *T. gondii* were recognized by rabbit antiserum against *N. caninum* KBA-2. Antigen spot 232 (69.4 kDa/p*I* 5.36), 245 (65.0 kDa/p*I* 5.34) and 248 (64.1 kDa/p*I* 4.97) were located at the same positions on 2-DE map of *N. caninum* KBA-2 and JPA1.

In part 3 of 2-DE maps, enolase (spots 34, 35 and 36) of *N. caninum* located at the same positions with spot T-101 (unidentified), 515 (enolase) and spot 514 (enolase) of *T. gondii*, at the range of pH between 5.3 and 5.7 with molecular weight between 42 and 57 kDa. Spot 516 (enolase) equivalents with spot 101 were also shown to locate at the compa-

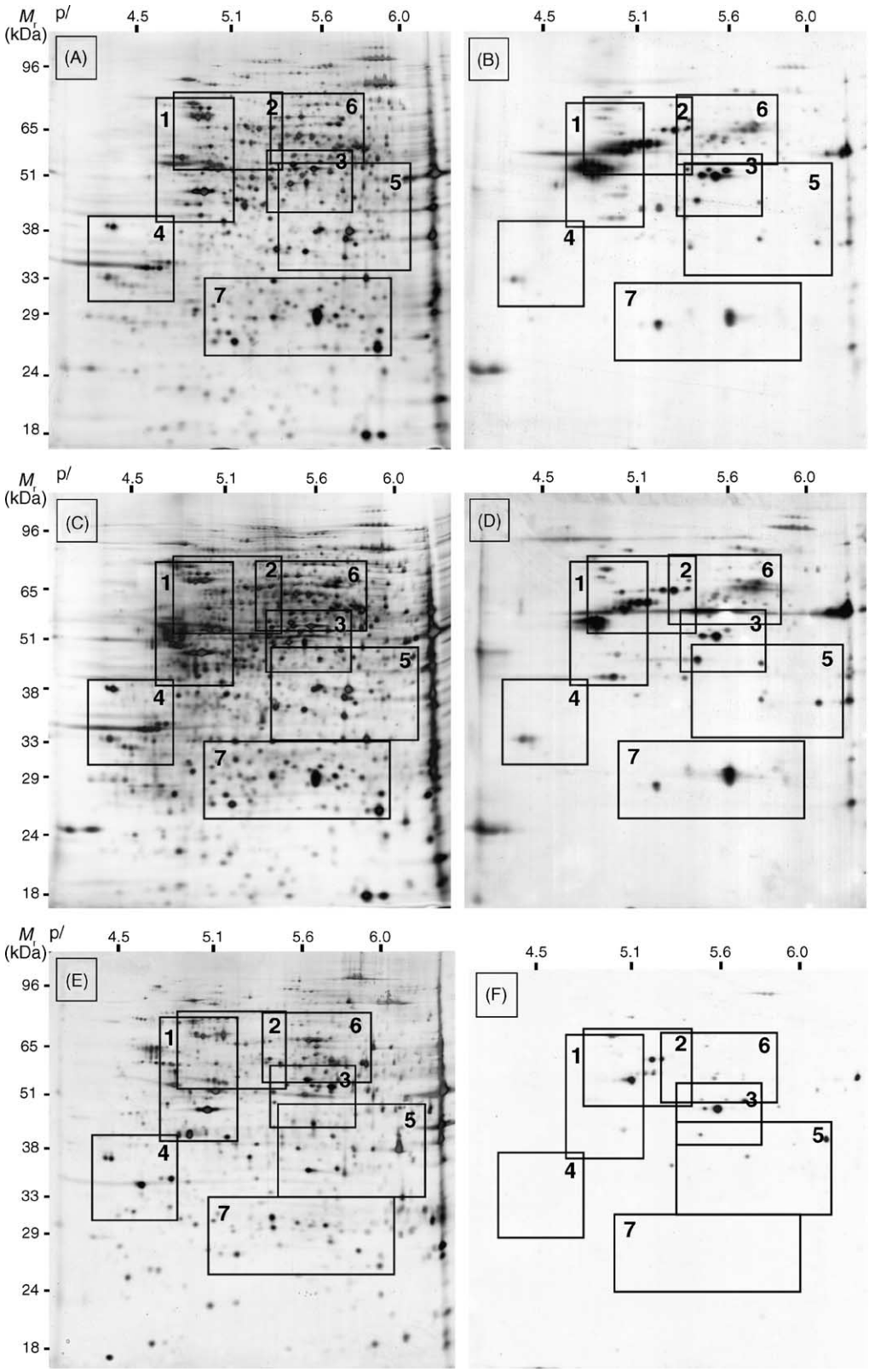

Fig. 1. 2-DE and its immunoblot profiles of *N. caninum* KBA-2 (A and B), JPA1 (C and D), and *T. gondii* (E and F). A total of 516 (A), 573 (C), and 419 (E) protein spots were detected on the 2-DE profiles, respectively. Of these, 94 (B), 91 (D), and 30 (F) spots were identified as antigenic spots on 2-DE immunoblot profiles using rabbit antiserum against *N. caninum* KBA-2. IEF was performed for a total of 86.1 kV h using pH 4– IPG strips (13 cm). SDS-PAGE was performed on a 12% gel which was then stained with silver nitrate. Separated proteins on the other gel were transferred to PVDF membrane and antigen spots were visualized using an ECL™ detection reagent. 2-DE profiles were divided into seven parts for comparative analysis.

Table 1
List of proteins identified on the 2-DE map of *N. caninum* and *T. gondii* tachyzoites after 2-DE followed by MALDI-TOF MS analysis

Spot number	Proteins	Number of matched peptides	Sequence coverage (%)	Theoretical M_r (Da)/p*I*	Accession number	Species
21	Subtilisin-like serine protease	9	13	93650/5.4	6119851	*N. caninum*
43	Lactate dehydrogenase	4	24	35549/6.0	1695772	*T. gondii*
45	Serine–threonine phosphatase 2C	9	26	36817/5.4	18076806	*T. gondii*
501	Heat shock protein 70	7	15	68530/5.2	5738969	*T. gondii*
503	Heat shock protein 70	13	24	73254/5.2	6682359	*T. gondii*
507	Nucloside-triphosphatase II	19	36	69586/5.7	2499221	*T. gondii*
508	Nucloside-triphosphatase I	7	14	69160/6.0	2499220	*T. gondii*
510	Heat shock protein 60	7	22	61014/5.8	5052052	*T. gondii*
511	Pyruvate kinase	7	21	57530/6.0	13928580	*T. gondii*
515	Enolase	6	24	48219/5.7	12619316	*T. gondii*
516	Enolase	10	26	48219/5.7	12619316	*T. gondii*
522	Fructose-1,6-bisphosphatase	11	35	42398/6.1	21715907	*T. gondii*
523	Serine–threonine phosphatase 2C	8	25	36817/5.4	18076806	*T. gondii*
524	Lactate dehydrogenase	4	24	35549/6.0	1695772	*T. gondii*

rable positions. Spots of 269 and 270 of *N. caninum* and those of T-102 and T-103 of *T. gondii* exhibited strong antibody responses against rabbit antiserum against *N. caninum* KBA-2 which were located similar M_r but different p*I* (Fig. 4).

Fig. 5 showed the protein profile of the part 4. The 14-3-3 protein homologue (N-12 and T-10) and N-11 and T-9 were found to be identical between *N. caninum* and *T. gondii* at pH ranges between 4.3 and 4.7 and molecular weight between 31

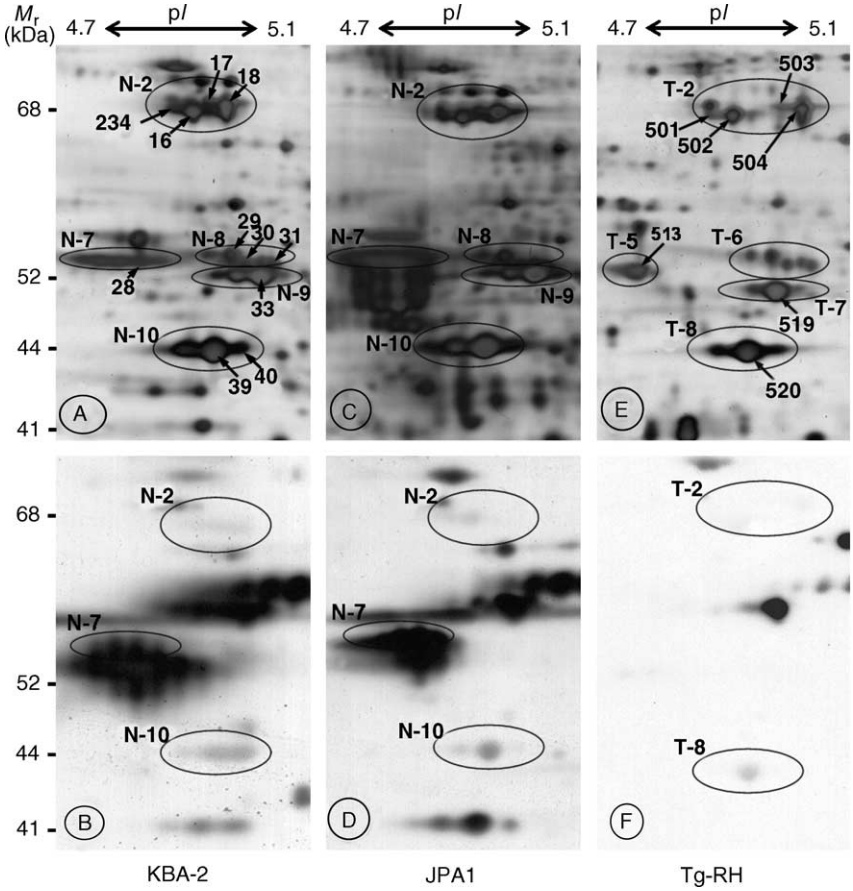

Fig. 2. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 1 on 2-DE maps at the range of pH 4.7–5.1 and molecular weight 41–76 kDa. 2-DE profiles of part 1 were showed similar protein spot patterns between *N. caninum* and *T. gondii* in terms of p*I* and M_r which were N-2 and T-2, N-7 and T-5, N-8 and T-6, N-9 and T-7, N-10 and T-8, respectively. Protein spots identified were HSP 70 (spots 16, 17, 18, 501, 502, 503 and 504), tubulin β-chain (spots 28 and 513), tubulin α-chain (spots 29, 30 and 31), putative protein disulfide isomerase (spots 33 and 519) and actin (spots 39, 40 and 520) using PMF analysis.

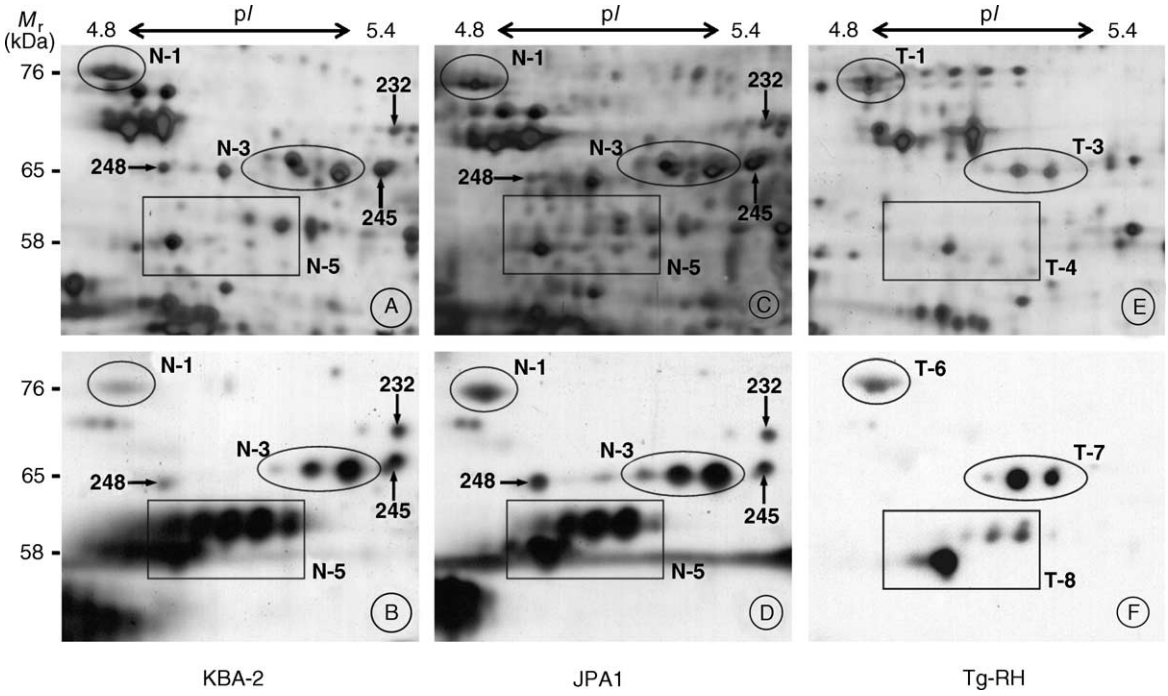

Fig. 3. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 2 on 2-DE maps at the range of pH 4.8–5.4 and molecular weight 52–76 kDa. 2-DE profiles of part 2 were showed similar protein and antigen spot patterns between *N. csninum* and *T. gondii* which were circle N-1 and T-1, N-3 and T-3 and box N-5 and T-4, respectively.

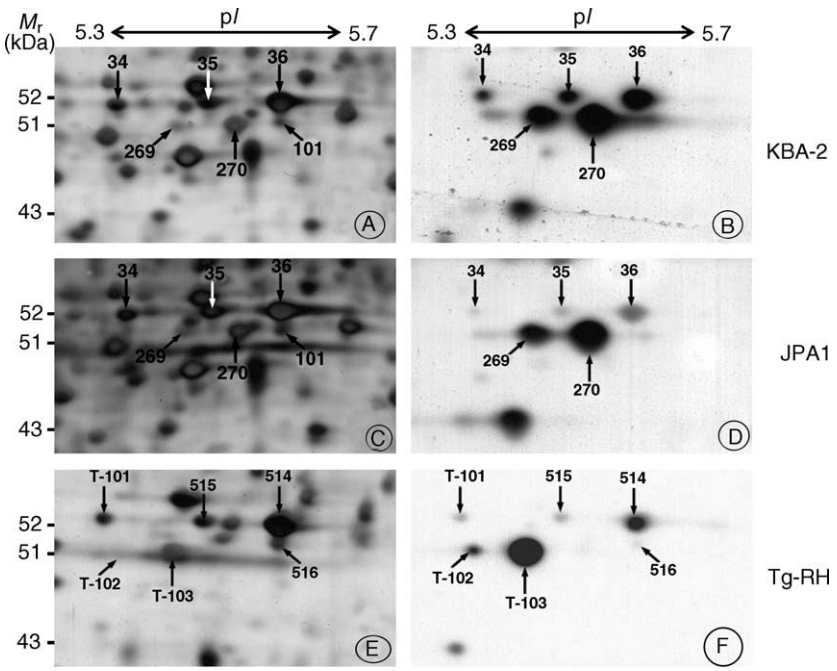

Fig. 4. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 3 on 2-DE maps at the range of pH 5.3–5.7 and molecular weight 42–57 kDa. 2-DE profiles of part 3 were showed similar protein and antigen spot patterns which were spots 34 and T-101, spots 35 and 515, spots 36 and 514 between *N. csninum* and *T. gondii*, respectively. Spots 269 and 270 of *N. caninum* and spots T-102 and T-103 of *T. gondii* on 2-DE profiles were showed antigenic response by rabbit-antiserum against *N. caninum* KBA-2 but they were located different p*I* and M_r. Spots 34, 35, 36, 514, 515 and 516 were identified as enolase using PMF.

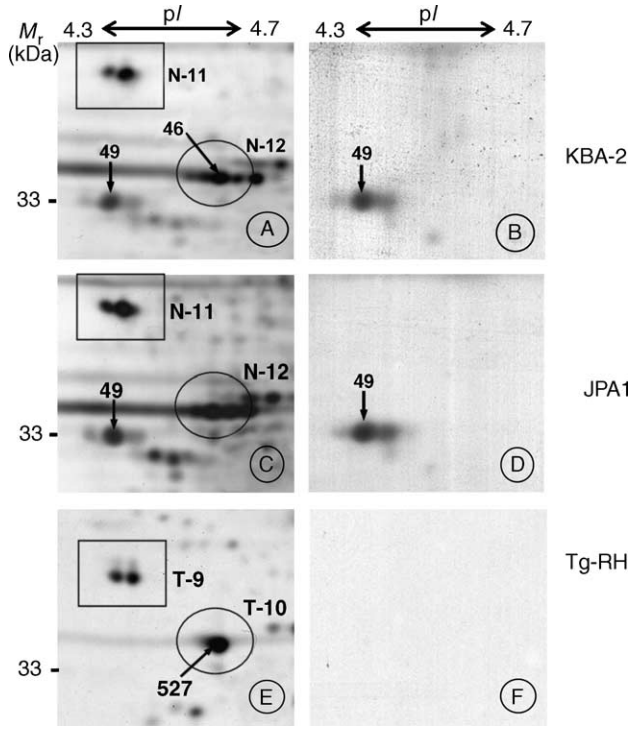

Fig. 5. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 4 on 2-DE maps at the range of pH 4.3–4.7 and molecular weight 31–40 kDa. Box N-11 and T-9 and 14-3-3 protein homologue (spots 46 and 527) were observed identical location but NCDG1 (spot 49), specific antigen of *N. caninum*, was exhibited only on 2-DE profiles of *N. canium*.

and 40 kDa. A specific antigen of *N. caninum*, NCDG1 (spot 49), was found to locate in this region. This protein clearly distinguished *N. caninum* from *T. gondii*.

In part 5 of 2-DE maps, fructose-1,6-bisphosphatase (spots 41 and 522), serine–threonine phosphatase 2C (spots 45 and 523) and lactate dehydrogenase (spots 43, 44, 524 and 525) were identified in *T. gondii* database using PMF analysis; however, these spots were observed at different positions on 2-DE profiles between *N. caninum* and *T. gondii* at pH ranges of 5.1 and 6.6 with molecular weight between 32 and 51 kDa. In addition, spots 41, 44 and 45 were specifically recognized on immunoblotting of *N. caninum* but not *T. gondii* (Fig. 6).

In 2-DE profiles of part 6, N-4 (marked by circle) consisted of three protein spots and contained NcSUB1 (spot 21). The spots could distinguish *N. caninum* from *T. gondii* since N-4 was not observed at pH ranges 5.2–6.4 and molecular weight between 52 and 70 kDa. NTPase (spots 22 and 23) of *N. caninum* were antigen proteins, but NTPase II (spot 507) and NTPase I (spot 508) of *T. gondii* were not recognized by rabbit antiserum against *N. caninum* KBA-2. Pyruvate kinase (spots 27 and 511) and heat shock protein 60 (spots 24 and 510) were located similar position on 2-DE profiles (Fig. 7).

NcGRA-2 (spot 50) and spot 88 (unidentified) were shown to be antigenic protein spots in *N. caninum* but these spots were not observed in *T. gondii* at pH ranges between 4.9 and 5.9 and molecular weight between 26 and 33 kDa (Fig. 8).

4. Discussion

Recent advances in 2-DE, mass spectrometry and bioinformatics have significantly improved the possibility of mapping and characterization of protein populations in medical and veterinary parasites [12,24–26]. In this study, we used proteomic approach for the comparative analysis of proteins expressed between *N. caninum* and *T. gondii* tachyzoites.

2-DE is one of the powerful and widely used tools for the analysis of complex protein mixtures [27,28]. Moreover, 2-DE combined with immunoblotting assay is allowed to find out many and distinct antigens compared with conventional SDS-PAGE (one-dimensional) and its immunoblotting analysis [29,30]. These methods have been employed successfully in characterizing the antigen profiles of *T. gondii* either with monoclonal antibody or patient's sera [13,14]. 2-DE map of *T. gondii* was reported [12] and detected average 630 (591–685) spots from 2-DE gel using pH 4–7 IPG strips (18 cm) stained with silver nitrate. On the contrary, average 469 (416–573) spots were detected from three 2-DE gels stained with silver nitrate using pH 4–7 IPG strips (13 cm). These differences could be mainly due to the different sample preparation, sample volume and gel size.

No morphological, biological, or molecular differences have been found between *N. caninum* isolates obtained from cattle and dogs [6,7,31,32]. *N. caninum* KBA-2 and JPA1 strains were isolated from very close geographical nations, such as Republic of Korea and Japan, respectively. They were exhibited almost the same 2-DE patterns, furthermore, antigen spots of KBA-2 and JPA1 on 2-DE immunoblotting profiles were located at the similar range of pH and molecular weight. On the other hand, comparison of 2-DE profiles between *N. caninum* and *T. gondii* was shown no sufficiently spot matching using analyzing software at the mode of programming matching. One of the reasons was suspected that different 2-DE profiles of *N. caninum* and *T. gondii* were not allowed to match the proteins in spite of locating some proteins at similar positions on 2-DE profiles [15].

Antigenic cross-reactivity between *N. caninum* and *T. gondii* were reported in ELISA test using crude *N. caninum* tachyzoites extract as coating antigen [33–37] and in immunohistochemical test using anti-*T. gondii* polyclonal and monoclonal antibodies [38,39]. Rabbit anti-*T. gondii* actin monospecific polyclonal antibody was also reacted with lysate antigen of *N. caninum* NC-1 isolate [40]. In the present study, some of protein spots on 2-DE profiles of *T. gondii* were reacted with rabbit antiserum against *N. caninum* KBA-2. Consequently, three antigenic proteins of *T. gondii*, including HSP70, actin and enolase, were believed as common antigens between *N. caninum* and *T. gondii*.

Micronemes and dense granules were known as secretary organelles of the Apicomplexan parasite which were playing a crucial role in invading host cells [41]. NcSUB1 of *N. caninum* was microneme protein which was homologous to TgSUB1 of *T. gondii* with 66% identity in the predicted catalytic domains [42,43]. NcGRA2 and NCDG1 (NcGRA7)

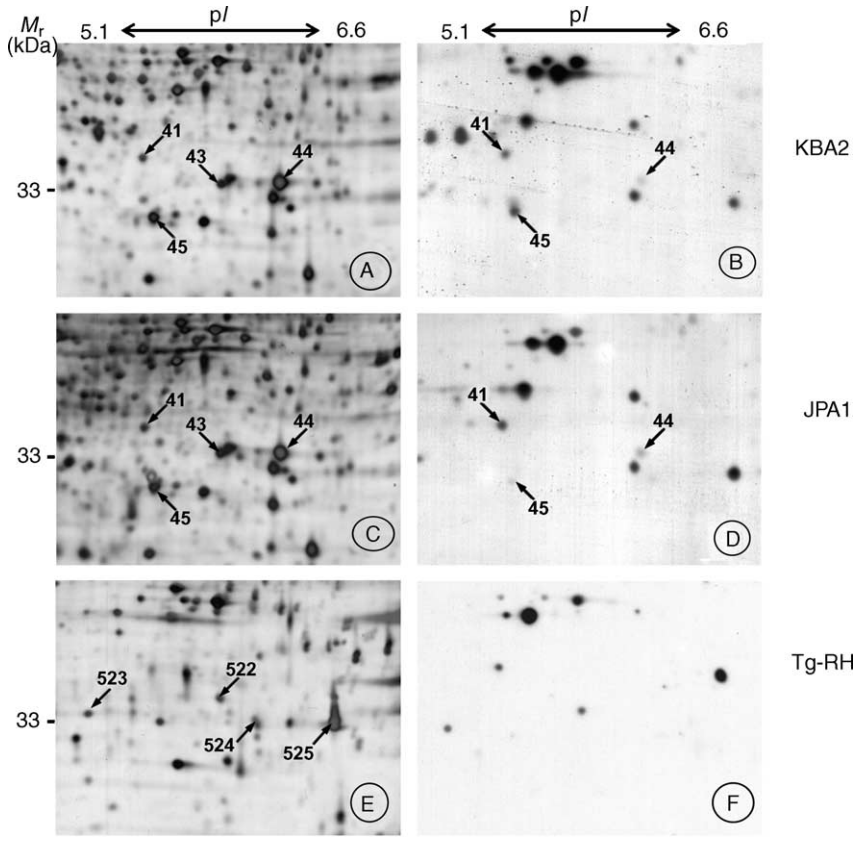

Fig. 6. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 5 on 2-DE maps at the range of pH 5.1–6.6 and molecular weight 32–51 kDa. Fructose-1,6-bisphosphatase (spots 41 and 522), serine–threonine phosphatase 2C (spots 45 and 523), and lactate dehydrogenase (spots 43, 44, 524 and 525) were identified with *T. gondii* database using PMF but different antigenic response by rabbit-antiserum against *N. caninum* KBA-2.

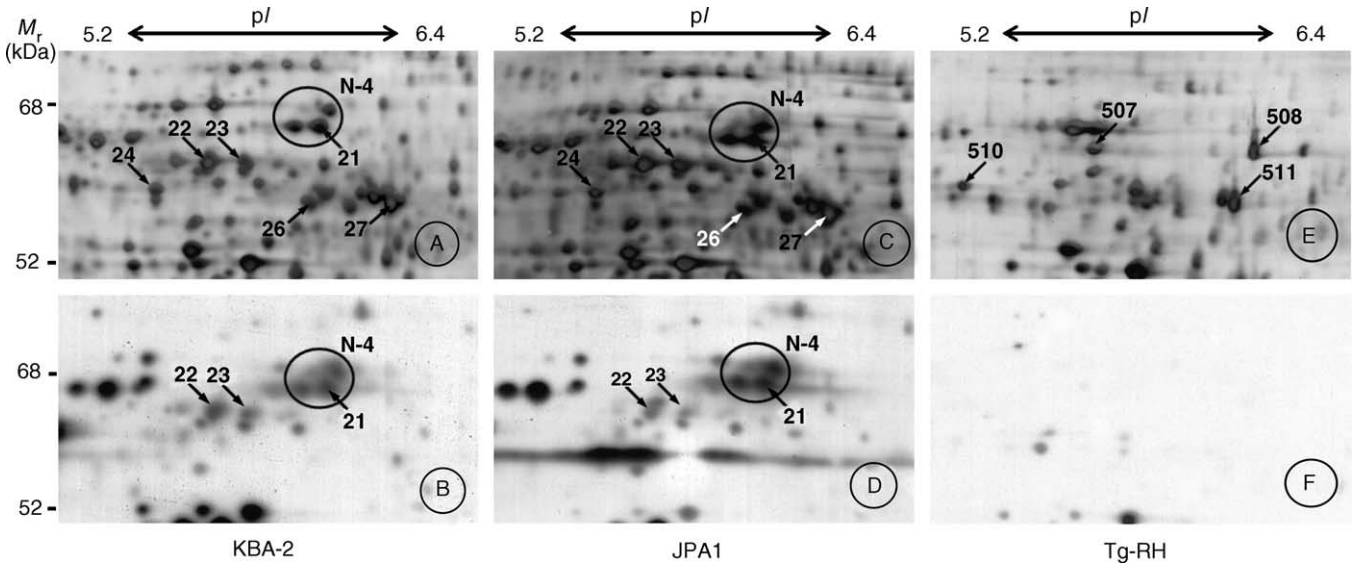

Fig. 7. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 6 on 2-DE maps at the range of pH 5.2–6.4 and molecular weight 52–70 kDa. Circle N-4 consist of three protein spots which were containing subtilisn-like serin protease (spot 21) was showed different protein profiles between *N. caninum* and *T. gondii* since these spots were not observed on 2-DE profiles of *T. gondii*. NTPase (spots 22 and 23) of *N. caninum* were antigen spots but NTPase (spots 507 and 508) of *T. gondii* were not. Pyruvate kinase (spots 27 and 511) and HSP 60 (spots 24 and 510) were located similar positions on 2-DE profiles.

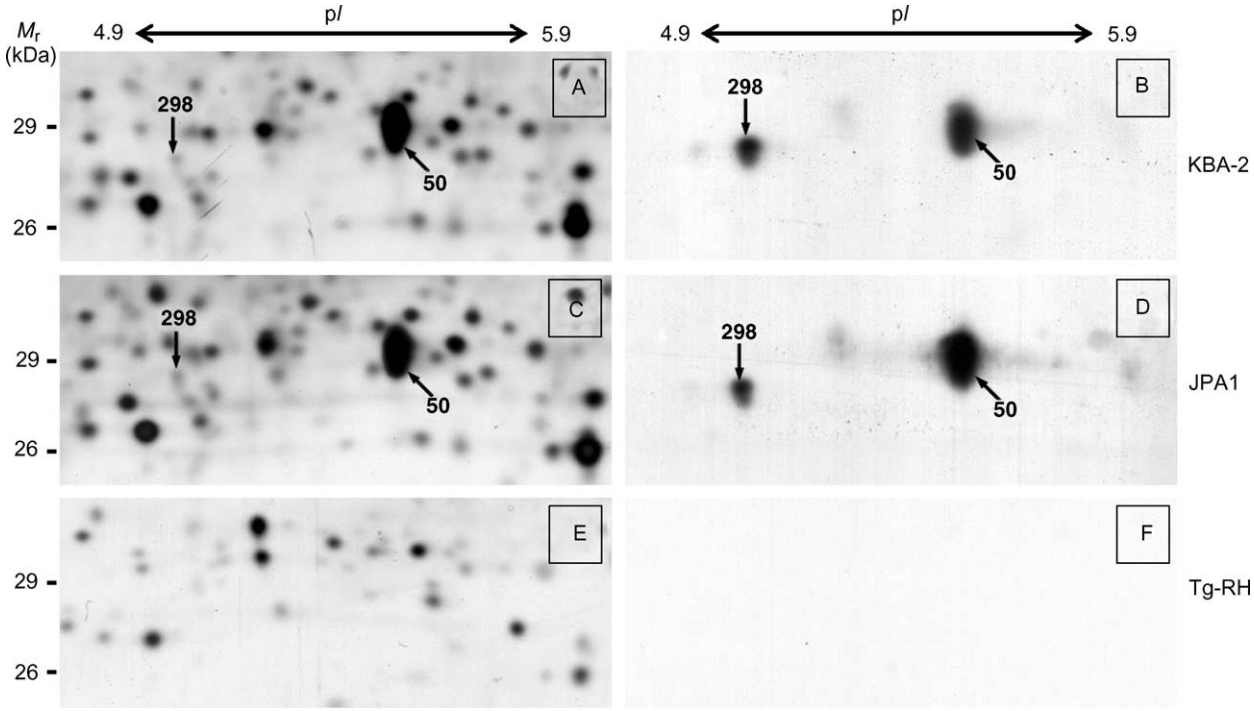

Fig. 8. Comparison of protein (A, C and E) and antigen (B, D and F) profiles of part 7 on 2-DE maps at the range of pH 4.9–5.9 and molecular weight 26–33 kDa. NcGRA-2 (spot 50) and spot 298 (unidentified) were showed different protein profiles between *N. caninum* and *T. gondii*.

were dense granule proteins, which were homologous to TgGRA2 and TgGRA7 of *T. gondii*, respectively. However, the dense granule proteins have relatively low homology in protein levels between NcGRA2 and TgGRA2, and between NCDG1 and TgGRA7 [40,44–46]. In this study, NcSUB1 and NcGRA2 were exhibited different 2-DE profiles between *N. caninum* and *T. gondii*. Moreover, NCDG1 of *N. caninum* and TgGRA7 of *T. gondii* were also located at different positions on the 2-DE maps [16]. In addition, NcSUB1, NcGRA2 and NCDG1 were suspected as possible species-specific proteins of *N. caninum* on the 2-DE maps on the basis of comparing 2-DE profiles between *N. caninum* and *T. gondii*.

In comparison of the predicted protein sequence, α-tubulin of *N. caninum* was identical to the α-tubulin of *T. gondii* [47]. Previous study, α-tubulins (spots 29–31) of *N. caninum* on 2-DE map was identified as conserved proteins using *T. gondii* database [16]. In the present study, α-tubulins of *N. caninum* were able to identify using *N. caninum* database since genomic sequence of α-tubulin was added in genomic database of *N. caninum* (data not shown). Protein spots of N-8 containing α-tubulins of *N. caninum* were exhibited similar protein spot distribution compared with T-6 of *T. gondii* (Fig. 2). As a result of this study, α-tubulins of *T. gondii* and *N. caninum* were highly suspected as conserved proteins.

NTPase (spots 22 and 23) of *N. caninum* is dense granule protein which was known to have similar enzymatic activity with NTPase I of *T. gondii*, and showed antigenic cross-reactivity with anti-*T. gondii* NTPase rabbit serum [48]. However, NTPase (spots 507 and 508) of *T. gondii* were not reacted with rabbit antiserum against *N. caninum* KBA-2 in the present study. Serine–threonine phosphatase 2C, fructose-1,6-bisphosphatase, and lactate dehydrogenase were shown as conserved proteins with *T. gondii* and *N. caninum* on 2-DE map. They were, however, exhibited different antigenic response on 2-DE immunoblot profiles with rabbit antiserum against *N. caninum* KBA-2. These proteins were needs further investigation to explain the different immunogenic properties by use of anti-toxoplasma serum.

As a consequence, the present studies were showed that there were very high similarities between *N. caninum* KBA-2 and JPA1, whereas the differences between *N. caninum* and *T. gondii* had been highly noticed on 2-DE profiles at pH range 4–7. Moreover, 2-DE coupled with immunoblot and mass spectrometry techniques were proved as very useful tools for finding specific antigens and the phylogenetic comparison either inter-species or closely related two apicomplexian parasites.

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