ALTERATIONS IN BRAIN CEREBRAL CORTEX PROTEOME OF RABIES-INFECTED CAT

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Abstract. Comparative proteome analysis using brain cerebral cortex tissues from cats and dogs infected with/without rabies virus were conducted using both two-dimensional gel-electrophoresis (2-DE) and 2-D fluorescence difference gel-electrophoresis (2D-DIGE) methods. The 2-DE gel images of all samples revealed >1,000 protein spots in each gel. Quantitative intensity analysis revealed the same overall protein pattern in certain regions of the gel, but the rabies-infected brains exhibited more protein spots than the non-infected controls. From approximately 880 protein spots detected by 2D-DIGE, 65 protein spots were increased and 46 were decreased. Eight of these protein spots were randomly selected and annotated by reference to previous known proteome data of rabid dog brains. They were similarly altered in both of the rabies-infected cats and dogs. A more detailed comparison of changes in proteomic profiles of brains between rabid cats and dogs should shed some light on the pathophysiological mechanism of rabies in domestic animals, as most rabies cases have been traceable to or believed to have originated from rabid dogs.

Keywords: brain proteomics, cat, cerebral cortex, 2-DE, 2D-DIGE, rabies

INTRODUCTION

The World Health Organization recognizes rabies, an infectious disease of the central nervous system (CNS), as the infectious disease with the highest fatality rate **(**WHO, 2013**)**. Dogs are the predominant animal with the highest mortality from rabies, followed by cats. The percent rabid cats among reported cases of rabies averages 3-5 according to the Ministry of Public Health annual report, Thailand, but they form potential reservoir that need to be better understood.

Clinical features of rabies can be classified as furious and dumb (Niezgoda *et al*, 2002). The furious form consists predominantly of profound agitation (excitement) and aggression. On the other hand, in the dumb form aggression may be completely lacking, but paralysis is predominant. Cats more often are reported as having furious rabies than dogs (Vaughn *et al*, 1963; Lobry, 1965). This may be because hyper-excitability and aggressiveness are less characteristic of normal cat behavior and are thus noted more readily in rabid cats. Cats that demonstrate an excitement phase become aggressive and scratch and bite without provocation.

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The clinical manifestations of rabies have been considered to be due to direct viral invasion of the nervous system. Negri bodies, which are dense masses of rabies viral proteins, are best seen in the cerebellum, hippocampus, brain stem, spinal cord and cerebral cortex (Matsumoto, 1975; Perl and Good, 1991). Studies *in vivo* using CVS-24-infected rats showed that there is progressive reduction in the expression of non-inducible housekeeping genes and late response gene, due to the suppression of cellular protein synthesis related to extensive synthesis of rabies virus mRNA. This occurs in association with induction of immediate-early-response genes in the hippocampus and cerebral cortex (Fu *et al*, 1993). In another study using subtraction hybridization, infection of mice with CVS-N2c rabies virus resulted in either up- or down-regulation of host genes, including those involved in regulation of cell metabolism, protein synthesis, growth and differentiation (Prosniak *et al*, 2001).

Recent developments in the field of proteomics have provided insights into the molecular basis of a number of diseases (Kellam, 2001; Maxell and Frappier, 2007). Comparative analysis of several models of rabies virus infection in the same affected tissues would be helpful in providing a better understanding of pathophysiological mechanisms of rabies infection. Moreover, comparison of proteome changes induced in different hosts following infection with typical rabies virus strains could provide valuable insights into rabies pathogenic effects.

Previous proteomic studies on rabies virus infection in cell cultures, and in murine and canine models have been conducted using conventional 2-dimensional gel-electrophoresis (2-DE) followed by mass spectrometry. Two-dimensional fluorescence difference gel-electrophoresis (2D-DIGE) is a modification of the classical 2-DE, in which two different protein samples and an internal standard are labeled with fluorescent dyes prior to performing 2-DE (Unlü *et al*, 1997; Alban *et al*, 2003). The technique enables analysis of differences in protein abundance in a single 2-D gel, so that all samples, even those separated on different gels can easily be compared and accurately quantified.

Little is known concerning the molecular mechanisms of feline rabies. There is no report demonstrating proteome of cat brain naturally infected with rabies and its changes in levels of proteins. Therefore, we investigated differences in brain cerebral cortex proteomes of cats with and without rabies using 2-DE and 2D-DIGE approaches.

MATERIALS AND METHODS

Sample collection

Brain tissues were obtained from cat carcasses submitted to the rabies diagnostic unit at Queen Saovabha Memorial Institute, Bangkok, Thailand. All brain specimens were diagnosed as being rabies infected by the fluorescent antibody test (Dean *et al*, 1996) and stored at -20°C until analyzed. Ten rabies-positive and 6 rabies-negative brain samples were studied. For comparison, 4 rabies-positive and 3 non-infected dog brains were investigated. This study was approved by the animal ethics committee of the Queen Saovabha Memorial Institute.

Protein extraction

Cerebral cortex tissues (1 g) were added to 1 ml of 2-D Protein Extraction Buffer-V (GE Healthcare, Uppsala, Sweden) containing a cocktail of protease inhibitors and sonicated (Sonics, Newtown, CT) on ice at 40% full power for five cycles of 20 seconds per cycle, followed by

centrifugation at 12,000*g* for 30 minutes at 4ºC. Protein concentration of the supernatant was determined using 2-D Quant Kit (GE Healthcare).

Conventional 2-DE

Protein samples (240 μg) were premixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol (DTT), 0.5% IPG buffer (pH 3-10) **(**GE Healthcare**)** and bromophenol blue dye (0.002%) to a final volume of 250 μl per sample. The solutions were spread onto IPG strip (linear pH gradient of 3-10, 13 cm in length; Immobiline DryStrip, GE Healthcare) and kept at room temperature for 10-15 hours. The first dimension isoelectric focusing (IEF) separation was conducted at 20ºC for 15,500 volt-hours using an Ettan IPGphor III IEF system (GE Healthcare). For the second dimension separation, the IPG strip was first incubated for 15 minutes in 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 1 % DTT, 2% SDS, 30% glycerol and 0.002% bromophenol blue, and then in the same buffer, but with DTT replaced with 2.5% iodoacetamide (IAA), for a further 15 minutes. Then the IPG strip was subjected to 12% SDS-PAGE at 20ºC at an initial 10 mAmp for 15 minutes, followed by 20 mAmp for approximately 4 hours (SE600 Ruby Vertical Electrophoresis Unit; GE Healthcare). Protein size markers (Genedirex, Taipei, Taiwan) also were included in the electrophoresis. Protein spots were stained with Coomassie brilliant blue G250 dye and images were recorded using an ImageMaster scanner (GE Healthcare).

2D-DIGE

Samples of extracted brain proteins were precipitated with 2-D Clean-Up Kit (GE Healthcare) and the pellets were resuspended in lysis buffer [30 mM Tris-HCl (pH 8.5), 7 M urea, 2 M thiourea and 4% CHAPS] at a final protein concentration of 5 μg/μl. Equal amounts of each sample (50 μg of protein) were pooled to generate an internal standard. The protein samples were labeled with CyDye™ DIGE Fluor dye (GE Healthcare) according to the manufacturer's instructions. Protein preparations were labeled with Cy3 or Cy5, and Cy2 was used to label the pooled internal standard proteins. In brief, 400 pmol of the required dye in 1 μl of anhydrous N,N-dimethylformamide were added per 50 μg of protein. The solution was kept on ice in the dark for 30 minutes, and then 1 μl aliquot of 10 mM lysine was added, followed by incubation for 10 minutes to terminate the labeling reaction. The Cy2-labeled internal standard sample and two samples (each labeled with either Cy3 or Cy5) were pooled and processed on IPG strip as described above with slight modifications. The IPG strip was 18 cm in length and 340 μl of IEF buffer consisting of 8 M urea, 4% CHAPS, 1% of IPG buffer and 60 mM DTT were used per strip. The first dimension separation IEF was performed for 24,000 volt-hours, and second dimension separation was performed in the dark by 12.5% SDS-PAGE (Ettan DALT*six* Electrophoresis Unit, GE Healthcare) at 10 mAmp for 30 minutes, followed by 40 mAmp until the dye front reached the bottom of the gel.

Image analysis

Image Master 2D Platinum software (GE Healthcare) was used for matching and analysis of visualized protein spots in Coomassie blue stained 2-DE gels. Parameters used were (i) minimal area of 10 pixels, (ii) smooth factor of 2.0, and (iii) saliency of 2.0. The 2D-DIGE gels were analyzed using a Typhoon Trio Variable Mode Imager (GE Healthcare) at a resolution of 100 dots cm^{-1}, with specific filters for Cy2 (filter BP40; 488-520 nm), Cy3

(filter BP30; 532-580 nm) and Cy5 (filter BP30; 633-670 nm). Analysis of CyDye-labeled protein spots were performed by DeCyder 2D 7.2 software (GE Healthcare) using Batch Processor, Difference in Gel Analysis (DIA), and Biological Variation Analysis (BVA) software modules. Batch Processing was accomplished by setting of 1,500 spots as the upper limit. In DIA, individual gel images were analyzed for spot numbers and spot intensities by comparing either a Cy3 or Cy5 image against Cy2 image from either rabies infected or non-infected samples. Spot patterns across different gels in the dataset were matched in the BVA module using Cy2-labeled spot pattern in each gel and analyzing for differences in abundance, normalized against Cy2 intensity for any given spot. Statistical analysis of protein variations was carried out using Student's *t*-test of volumes (area x intensity) of matched spots with > 2-fold changes and statistical significance is when *p*-value is < 0.05.

RESULTS

Proteins extracted from cerebral cortex of 10 rabid and 9 normal control cat brains were compared using both conventional and 2-DE and 2D-DIGE. Using 2-DE, comparison of all Coomassie blue-stained gel images revealed > 1,000 protein spots in each gel and that the cerebral cortex of rabies-infected cat brains exhibited more protein spots than the normal cat samples at similar protein amounts, but both types of tissues were found to have the same overall protein pattern in certain regions of the gel (Fig 1). The majority (>50%) of the protein spots from rabid samples were clustered in the higher molecular mass (>38 kDa) region of the gel, and there was a clustering of acidic proteins of 35-100 kDa. The 2-DE patterns of cerebral cortex proteins from rabid dog brains were similar to those of rabid cats (data not shown).

In the 2D-DIGE assay, gel images were matched and individual protein spots were normalized with respective to the same spiked protein mixture added to each sample prior to conducting 2-DE. From approximately 880 protein spots visualized in each 2D-DIGE gel, quantitative intensity analysis revealed 65 (7.4%) and 46 (5.2%) protein spots significantly increased and decreased, respectively ≥2-folds in rabid compared to normal control cat cerebral cortex (Fig 2). Cerebral cortex proteins from 4 rabid dog brains also were analyzed by 2D-DIGE (data not shown).

A number of protein spots whose levels are significantly altered in rabid cats shown by 2-DE and 2D-DIGE were randomly selected (numbered spots in Figs 1 and 2) for comparison with those from rabid canine samples. There were identical changes observed among rabid cat and dog samples (data not shown). We randomly selected eight protein spots and tentatively identified them based on previously published 2-DE map of cerebral cortex proteins of rabid dog brains (Table 1) (Thanomsridetchai *et al*, 2011).

DISCUSSION

Comparison of complex protein mixtures using proteomics can be achieved by 2-D technique, and the separated proteins can be visualized by Coomassie blue dye staining or by pre-labeling with a variety of fluorescent dyes. The former technique has certain disadvantages, such as a lack reproducibility. With 2D-DIGE at least three fluorescent dyes are commercially available and therefore two samples and an internal standard (internal reference) can

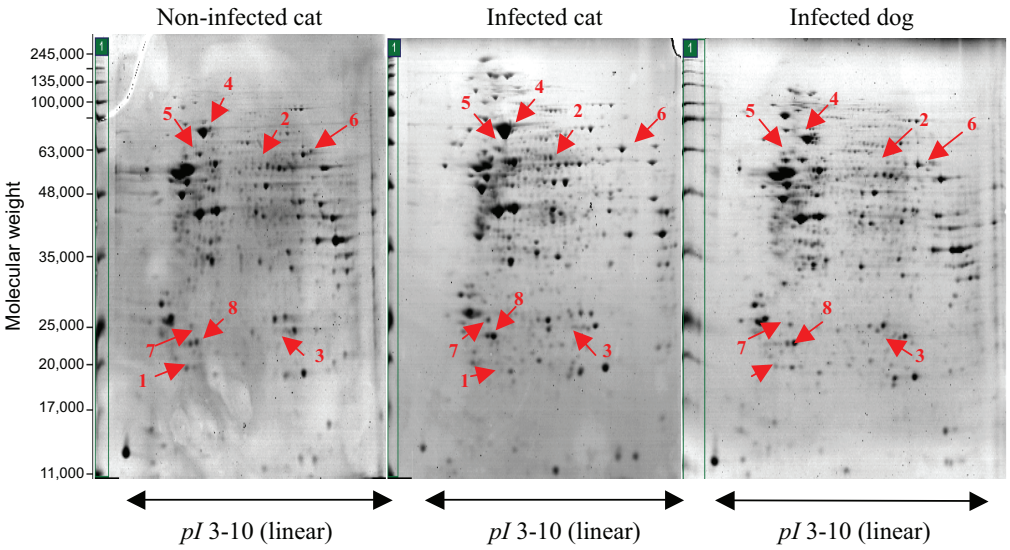

Fig 1–Representative 2-DE gel images of brain cerebral cortex proteome of rabies-infected cat and dog and non-infected cat. A total of 240 μg of protein were resolved and visualized with Coomassie blue dye. Spots are numbered according to Table 1.

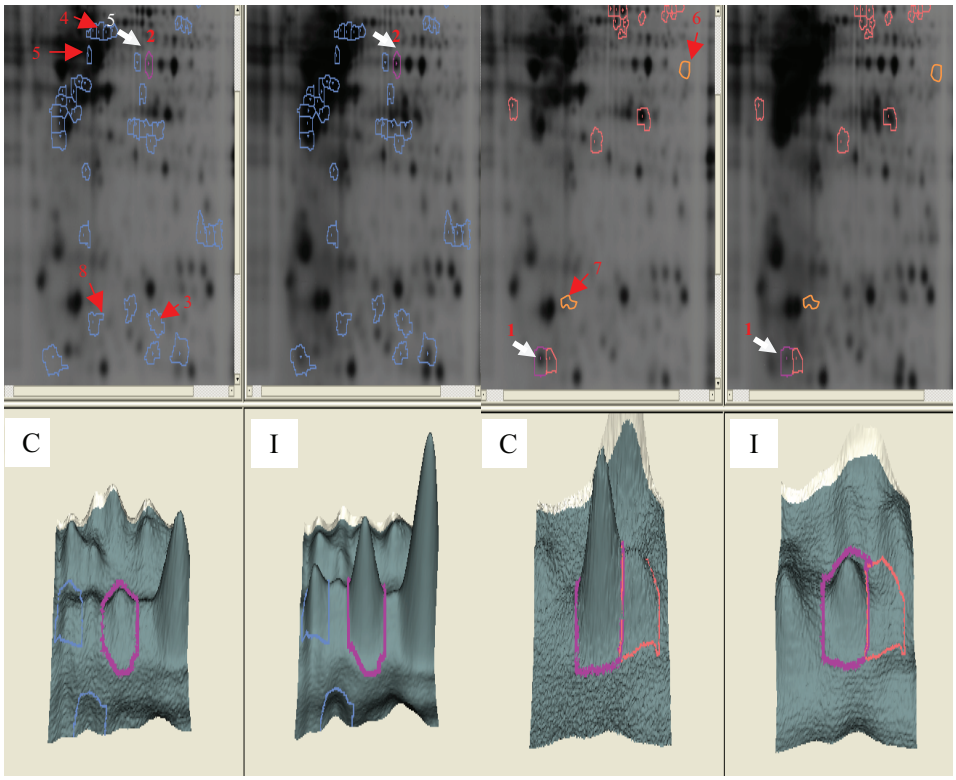

Fig 2–2D-DIGE images and representative 3-D simulation views of brain cerebral cortex proteins from control (C) and rabies-infected (I) cats. Proteins that are increased and decreased relative to control are shown in blue and orange circles respectively. Spots are numbered according to Table 1.

Table 1
Representative differentially expressed in brain cerebral cortex of rabies-infected cat.

Spot no.[a]	Predicted protein[b]	Observed MW (kDa)/*pI*	Identical changes in dog brain proteins (spot no.)[b]
1	Cytoskeleton (TUBB2B protein)	20.89/4.85	Decreased (#1000)
2	Cytoskeleton (Keratin 1)	62.02/6.78	Increased (#1040)
3	Immune regulatory (Interferon alpha 4)	23.45/6.93	Increased (#107)
4	Immune regulatory (SARM 1 protein)	70.30/5.93	Increased (#1359)
5	Neuron-specific (Dihydropyrimidinase related protein-2)	64.62/5.36	Increased (#382)
6	Apoptosis-related (Cytochrome P450 2B12)	59.61/8.28	Decreased (#192)
7	Anti-oxidants (Hypoxanthine phosphoribosyltransferase 1)	25.27/5.34	Decreased (#791)
8	Anti-oxidants (Peroxiredoxin 2)	23.16/5.46	Increased (#888)

[a]Protein spots numbered in Figs 1 and 2; [b]From Thanomsridetchai *et al* (2011).

be analyzed in the same gel. The internal standard comprises pooled aliquots from all the samples within a particular set of experiments and is included in each sample to be electrophoresed. As identical proteins labeled with different dyes will migrate to the same position in the 2-D gel, amounts of the differentially tagged proteins can be quantitated and compared *in situ*, allowing the subtle changes in protein expression levels to be detected accurately.

In order to identify cat cerebral cortex proteins whose expression levels were altered as the result of the neuropathogenesis caused by rabies infection, we initially compared the 2-DE proteome profiles of cats with those of dogs, which yielded similar images by visual comparison. However, using 2D-DIGE, we were able to quantify each protein spot and determine those significantly altered as the result of rabies infection. Comparison of these cat protein spots with those from rabid canine brain cerebral cortex analyzed in parallel, 8 proteins were annotated using a previously published profile (Thanomsridetchai *et al*, 2011). These proteins play roles in cytoskeleton network, immune function, neurons and as anti-oxidants. The disturbances to the cytoskeleton integrity of the neurons have been postulated as a possible basis for neuronal dysfunction (Zandi *et al*, 2013). In addition, alterations in expression of regulatory and structural proteins may have potential roles in rabies pathogenesis. Previous neuronal studies have demonstrated that fatal rabies may result from neuronal dysfunction rather than structural damage (Tsiang, 1982; Ceccaldi *et al*, 1993; Fu and Jackson, 2005). There are many signature proteins known to be associated with ra-

bies from proteome analysis in different models of rabies infection, but not much identical changes were observed (Dhingra *et al*, 2007; Thanomsridetchai *et al*, 2011; Wang *et al*, 2011; Kluge *et al*, 2013; Venugopal *et al*, 2013; Zandi *et al*, 2013).

Cases of rabies in domestic animals other than dogs have been traceable or believed to have originated from exposure to rabid dogs (Kasempimolporn *et al*, 2004, 2008). Comparing expression levels of proteins between rabid and normal hosts might lead to identification of candidate biomarkers for rabies in domestic animals. It is highly likely that the rabid cats used in this study were infected with different viral strains and with different doses, but they showed protein spots that were similarly altered in cerebral cortex of infected brains. The significance of similar or identical changes of some protein spots in cats experimentally infected with the same doses of the same viral strains and also in different location of the CNS tissues should be further studied to lend support of the present findings, as it indicates that such proteins may be under the influence of similar control mechanisms.

There were limitations to the present study, including its inability to use mass spectrometry for protein identification and lack of an in-depth analysis of the cat rabies proteome. Some of the proteins may appear at multiple positions on the gels, consistent with the presence of different post-translationally modified forms. We will further validate some of protein spots by western blot analysis and/or apply other proteomic techniques such as reversed-phase high performance liquid chromatography in an effort to pursue this issue and to strengthen this set of data.

In conclusion, an understanding of the basis of rabies virus neurovirulence is emerging from studies using a variety of animal models. The host reaction to invasive and attenuated strains of rabies virus might be different. Identical changes in cellular proteome profiles observed among cats and dogs naturally infected with rabies could provide a reference in future research on rabies virus infection, leading to a better understanding of the pathophysiology of rabies.

ACKNOWLEDGEMENTS

The study was supported by the Queen Saovabha Memorial Institute. The authors are grateful to the anonymous reviewer for advice on revising the manuscript.

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