Proteome Analysis of Cerebrospinal Fluid in Healthy Beagles and Canine Encephalitis

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ABSTRACT. We performed proteomics analysis of the cerebrospinal fluid (CSF) of healthy dogs and dogs with meningoencephalitis of unknown etiology (MUE). By comparing two-dimensional electrophoreses (2DE), an upregulated spot was found in MUE dogs. This protein was identified as a neuron-specific enolase (NSE) by analysis with MALDI-TOF mass spectrometry. In comparing dot blots using an antibody against NSE, the NSE levels in the CSF of MUE dogs was significantly higher than that of the controls. NSE is a diagnostic marker of neuroendocrine tumors, brain injury and spinal cord trauma in humans. It seems that the NSE concentration in the CSF is increased by cellular destruction in canine encephalitis. Though elevation of NSE may not be specific in canine encephalitis because the NSE level was increased in other CNS diseases, further study including measurement with serum is necessary.
KEY WORDS: 2DE, canine, CSF, encephalitis, proteome.

Dogs are affected by various diseases of the central nervous system (CNS). However, the origin of some conditions, such as CNS inflammatory disease, is unknown. The development of cures for CNS diseases will require more in-depth investigations to determine the underlying causes. Meningoencephalitis of unknown etiology (MUE) is one of the most common clinical diagnosis in canine inflammatory CNS disease. The terminology *meningoencephalitis of unknown etiology* is proposed to describe dogs with a CNS inflammatory disease that lacks a histopathological diagnosis [19]. Necrotizing meningoencephalitis (NME), granulomatous meningoencephalomyelitis (GME) and necrotizing leukoencephalitis (NLE) are included in MUE. Because a tissue diagnosis during the lifetime of a patient is difficult in current veterinary medicine, we tend to diagnose patients with MUE until postmortem histopathological examination.

Cerebrospinal fluid (CSF) is the only body fluid that surrounds the brain, and analysis of CSF can provide direct information regarding the physiological condition of the brain. This is therefore often performed as part of the diagnostic process for various neurological diseases. Studies of CSF have been performed in relation to various canine CNS diseases. However, there have been no comprehensive analyses of the canine CSF proteome. In humans, analyses of the CSF proteome have been used to search for diagnostic markers in patients with multiple sclerosis, Guillain-Barré syndrome and Alzheimer's disease [1, 4, 5, 11, 16, 17]. The search for biomarkers of neurological diseases in dogs will require proteomic analyses of CSF in normal dogs. We aimed to determine a characteristic CSF protein pattern by comparing control and MUE dogs with 2DE. We also compared the level of a protein that showed differential expression in CSF.

MATERIALS AND METHODS

Sample collection: CSF was taken by cisternal puncture from 15 healthy beagles (age: 4.9 ± 2.8; average ± SD) under general anesthesia and centrifuged at 1,000 × *g* for 8 min at 4°C to eliminate cells and insoluble material, and then the supernatant fluid was stored at −70°C until analysis.

Twenty-five dogs were diagnosed with MUE based on clinical findings and findings from magnetic resonance imaging (MRI) and routine CSF examinations as follows: (1) focal or multifocal neuroanatomical localization, (2) negative blood and/or CSF infectious disease titers, (3) CSF pleocytosis and/or increasing of protein level (5 cells/mm^3 and/or 25 mg/d*l*), (4) MRI of the brain consistent with focal or multifocal disease (some cases had formed cavitation), (5) fundoscopic and neurological examination consistent with optic neuritis (blindness with normal electroretinogram in the absence of imaging evidence of thalamic or cerebral disease). CSF was taken as described above. The MUE cases comprised ten Pugs, five Shih-Tzus, four Yorkshire Terriers, two Chihuahuas, two Malteses and two Pekingeses (age: 3.0 ± 2.7; average ± SD).

Two-dimensional electrophoresis (2DE): To concentrate proteins and remove salt, all control samples and some

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encephalitis samples were centrifuged with Amicon Ultra® (Millipore Corporation, Billerica, MA, U.S.A.) at 4°C. Protein concentration was determined in all concentrated samples by a 2-D Quant kit (GE Healthcare, Buckinghamshire, U.K.).

Isoelectric focusing (IEF) was carried out with a PRO-TEAN® IEF Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For analytical runs, 40 μg of protein (100 μg for preparative runs) was mixed with a rehydration solution containing 8 M urea, 2% CHAPS, 0.5% carrier ampholytes (Bio-Lyte® 3/10 buffer, Bio-Rad), 18 mM DTT and a trace of bromophenol blue in a total volume of 200 μl and applied to immobilizing pH gradient gels (ReadyStrip™ IPG Strips, 7 cm, 3–10 NL, Bio-Rad). After passive rehydration for 14 hr at 20°C, IEF was performed for both analytical and preparative runs as follows: rapid 250 V for 1 hr; linear 8,000 V for 2 hr and rapid 8,000 V for 80,000 V-hr. Focused strip gels were reduced by equilibrating for 30 min in equilibration buffer containing 64.8 mM DTT and then alkylated by equilibrating for 30 min in 135 mM iodoacetamide. SDS-PAGE was performed according to the Laemmli method [10], with slight modifications, on 13% gels at a constant current of 5 mA/gel at 4°C until the bromophenol blue dye front reached the bottom of the gel. After SDS-PAGE, analytical gels were silver stained (Dodeca™ Silver Stain Kit, Bio-Rad), and preparative gels were stained with CBB.

In-gel digestion and MALDI-TOF MS analysis: The protein spots observed in preparative gels that corresponded to spots observed in analytical gels were excised and then destained with 50 mM NH_4HCO_3 and 50% methanol. After washing with Milli-Q water, the gel pieces were dehydrated with 50 mM NH_4HCO_3 and 50% acetonitrile (AN) and then completely dehydrated with 100% AN. Porcine trypsin (50 ng; Promega, Madison, WI, U.S.A.) dissolved in 5 μl of 50 mM NH_4HCO_3 was added to each gel plug along with 5 μl of 100 mM Tris-HCl, pH 8.8. Proteins were digested for 15 hr at 37°C, and the resulting peptides were eluted from the gel plugs at room temperature with 50 μl of 50% AN/0.1% trifluoroacetic acid (TFA) and dried in a vacuum centrifuge down to a total volume of 20 μl. The samples were then purified by binding them to a pipette tip (ZipTip®, Millipore Corporation) followed by elution with 50% AN/0.1% TFA and 90% AN/0.1% TFA directly onto the MALDI target. Immediately after samples were applied to the target, 0.5 μl of 25% α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany), 50% AN and 0.1% TFA was stratified and mixed with each sample on the MALDI target.

Peptide mass fingerprint spectra were recorded by MAL-DI-TOF MS (Autoflex®, Bruker Daltonics). The proteins were identified from the mass spectrometry data using the Mascot search program (www.matrixscience.com). Database search results were manually checked by comparison with the nominal mass and calculated pI value. The protein spot that showed differential expression between the control and encephalitis dogs was excised from the 2DE gels and analyzed by the same method.

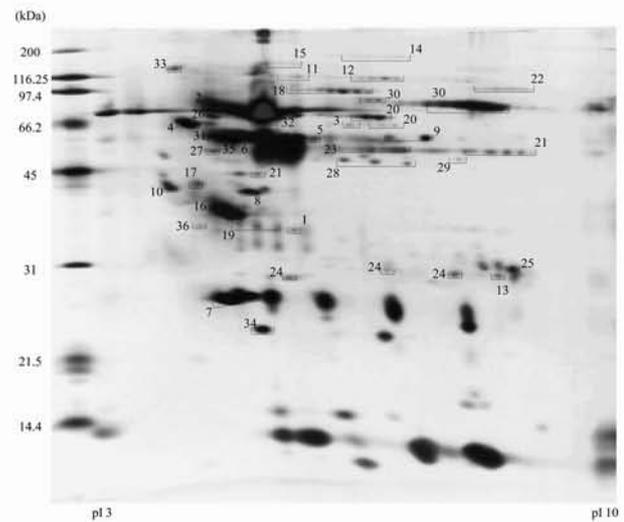*2DE Western blotting (WB)*: Control and MUE CSFs

Fig. 1. 2DE map of canine CSF proteins. Protein spots are numbered according to the Mascot search results. Proteins were electrofocused in the first dimension on IPG ReadyStrips (11 cm, 3–10 NL), and after 2DE, the resultant gels were silver stained.

were separated by an isoelectric electrophoresis system (PROTEAN® IEF Cell, Bio-Rad). Ten micrograms of CSF proteins were mixed with the rehydration solution in a total volume of 125 μl and applied to immobilizing pH gradient gels (ReadyStrip™ IPG Strips, 7 cm, 3–10 NL, Bio-Rad). After passive rehydration for 14 hr at 20°C, IEF was performed as follows: rapid 250 V for 30 min; linear 4,000 V for 1 hr and rapid 4,000 V for 20,000 V-hr. Focused strip gels were reduced by equilibrating for 30 min in equilibration buffer containing 64.8 mM DTT and then alkylated by equilibrating for 30 min in 135 mM iodoacetamide. SDS-PAGE was performed on 10% gels at a constant current of 3 mA/gel at 4°C until the bromophenol blue dye front reached the bottom of the gel, and then transferred onto PVDF membranes (ATTO, Tokyo, Japan).

The membranes were blocked with 2.5% skim milk in PBS. WB analysis was performed using mouse anti-neuron specific enolase monoclonal antibody (diluted at 1:100; GenWay Biotech, San Diego, CA, U.S.A.) and HRP-conjugated anti-mouse IgG antibody (1:10,000; Pierce Biotechnology, Rockford, IL, U.S.A.). Immunoreactions on the membranes were detected using ECL™ Western blotting detection reagents (GE Healthcare) and scanned with an Ez-Capture II imaging system (ATTO).

Dot blotting: Ten microliters of CSF samples and 10 μl of human recombinant NSE (diluted at 1:200–20,000; ATGen, Sung Nam, Kyung Ki Do, South Korea) were spotted on PVDF membranes using Immunodot® (ATTO). Antigen-antibody reactions were performed as described above with same the antibodies and same concentrations. Immunoreactive spots on the membranes were detected using ECL™ Western blotting detection reagents (GE Healthcare) and scanned with an Ez-Capture II imaging system (ATTO). Quantification of spots was performed using CS Analyzer

Table 1. Canine CSF proteins identified in this study

No.[a]	Protein identification result[b]	Score[c]	pI[d]	% Sequence coverage[e]	Nominal mass[f]
1	Albumin	78	5.36	16	67857
2	Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein)	203	5.38	41	56264
3	Alpha-2-antiplasmin precursor (Alpha-2-plasmin inhibitor) (Alpha-2-PI) (Alpha-2-AP)	102	6.68	31	55147
4	Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Alpha-2-Z-globulin) (Ba-alpha-2-glycoprotein)	70	5.64	24	39729
5	Angiotensinogen precursor	139	5.9	32	53578
6	Antithrombin-III precursor (ATIII)	102	6.58	36	53712
7	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	191	5.28	75	30163
8	Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV)	206	5.56	56	46304
9	Beta-2-glycoprotein 1 precursor	229	8.51	52	39689
10	Clusterin precursor	100	5.65	35	52327
11	Complement C3 precursor	110	6.95	15	175356
12	Complement factor B precursor (C3/C5 convertase) (Properdin factor B) (Glycine-rich beta glycoprotein) (GBG) (PBF2)	143	7.18	34	87635
13	Complement factor D precursor (C3 convertase activator) (Properdin factor D) (Adipsin)	138	9.27	44	31951
14	Complement factor H precursor (H factor 1)	119	6.28	18	101834
15	Contactin 1 isoform 2 precursor isoform 1	77	5.69	16	112693
16	Full=Apolipoprotein E; Short=Apo-E	149	5.2	40	35332
17	Full=SPARC; AltName: Full=Secreted protein acidic and rich in cysteine; AltName: Full=Osteonectin; Short=ON; AltName: Full=Basement-membrane protein 40; Short=BM-40; Flags: Precursor	83	4.71	33	35446
18	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)	160	6.3	38	81353
19	Haptoglobin heavy chain, HpH chain	110	5.8	51	27269
20	Hemopexin	131	6.88	38	52047
21	Hypothetical protein XP_533132	108	5.24	38	42053
22	Immunoglobulin gamma heavy chain B	126	8.52	29	52553
23	Immunoglobulin gamma heavy chain C	114	6.16	31	52779
24	Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related 14.1 protein) (Immunoglobulin omega polypeptide) (Lambda 5) (CD179b antigen)	86	8.84	52	15118
25	Kallikrein 6 precursor (Protease M) (Neurosin) (Zyme) (SP59)	114	7.57	35	27747
26	Kininogen 1	187	5.58	44	49400
27	Leucine-rich alpha-2-glycoprotein 1	104	6.17	35	38390
28	Pigment epithelium-derived factor	119	8.69	46	44293
29	Procollagen C-proteinase enhancer protein precursor (PCPE) (Type 1 procollagen COOH-terminal proteinase enhancer) (Type 1 procollagen C-proteinase enhancer protein)	123	6.82	37	49353
30	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin)	186	6.07	31	80155
31	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	83	5.58	32	46505
32	Serum albumin precursor	128	5.52	45	70556
33	SPARC-like protein 1 precursor (High endothelial venule protein) (Hevin) (MAST 9)	107	4.61	38	70706
34	Transthyretin precursor (Prealbumin) (TBPA) (TTR) (ATTR)	82	6.42	51	15972
35	Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDB)	91	5.2	31	54536
36	Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein) (Zn-alpha-2-GP)	144	4.81	52	36060

a) Numbers correspond to spot numbers in Fig. 1. b) Proteins identified by Mascot search. c) The protein score is $-10*Log(P)$, where P represents the probability that the observed match is a random event. Protein scores greater than 67 are significant ($P<0.05$). d) The isoelectric point expected for a protein with the sequence returned by the database search. e) Refers to the percent of the protein sequence identified by mass spectra. f) The nominal mass is obtained by summing the integer masses of the most abundant naturally occurring stable isotopes of the elements constituting the protein.

3.0 (ATTO). The multiplication value was calculated from the quantity of the luminescence and the area by CS Analyzer. We made a calibration curve from the multiplication value of the luminescence of the recombinant NSE and calculated the NSE concentration. Differences in concentration between the control group and the MUE group were subjected to the Mann-Whitney U test. A P value <0.05 was considered significant.

RESULTS

2DE: Ten CSF samples were prepared from healthy beagles, the proteins were separated by 2DE and the resultant gels were silver-stained. A total of 134 protein spots were detected on each gel. From these 134 spots, a total of 97 proteins were identified by MALDI-TOF MS and Mascot searching (Fig. 1). The 97 proteins that were identified were classified into 36 classes, as shown in Table 1. Each of the proteins identified in this study were found in all of the con-
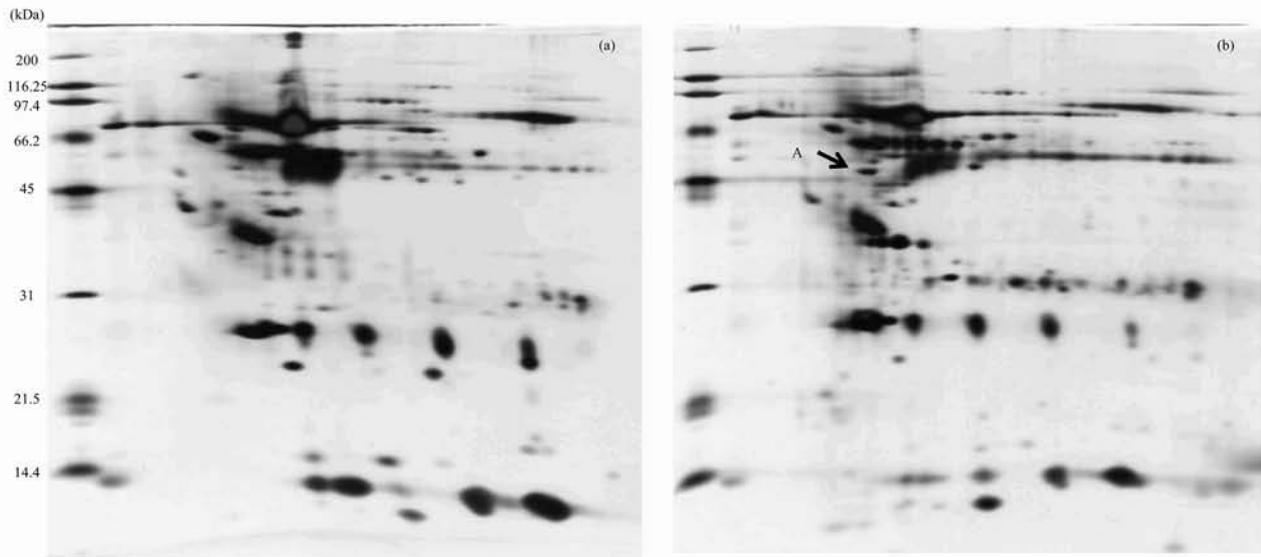
Fig. 2. Control (a) and MUE subject (b) 2DE. Some upregulated spots were detected in MUE subject CSF. Spot A was upregulated in all MUE animal CSFs.
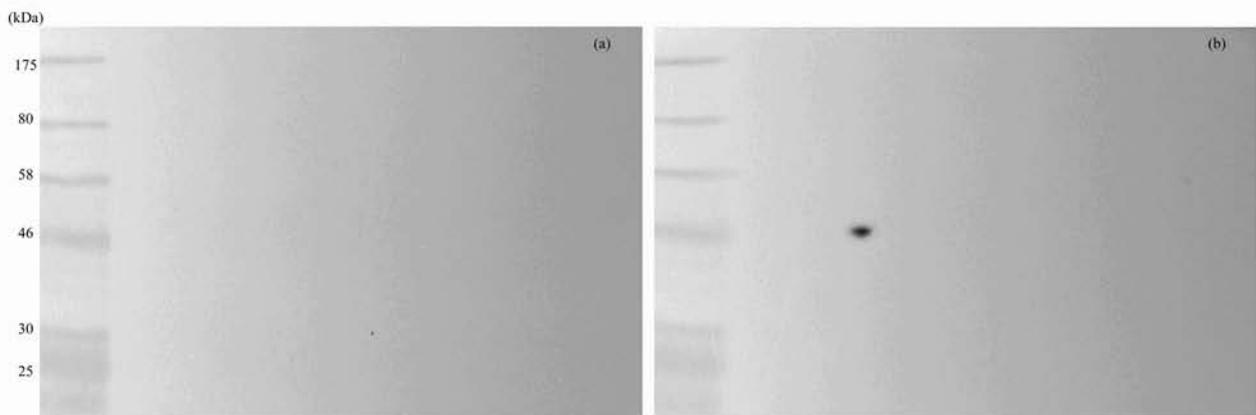

Fig. 3. Result of 2DE-WB. The immunoreactive spot was detected as a unique spot in MUE subject CSFs (b). This spot was consistent with the 2DE gels.

trol CSF samples.

Typical silver-stained 2DE gels of CSF from diseased animals compared with the control group are shown in Fig. 2a and 2b. Some protein spots were found to differ significantly in the gels between the two groups. Spot A was upregulated in all diseased animals as compared with the controls.

MALDI-TOF MS analysis: The results of the PMF spectra analysis of spot A are shown in Table 2. The spot was identified as a canine neuron-specific enolase. The top score and sequence coverage were 214 and 56%. The nominal mass and pI values of NSE were 47,499 and 4.98. These data corresponded with the 2DE gels.

2DE-WB: The results of 2DE-WB are shown in Fig. 3. The spot was detected in the same position on 2DE gels. The spot was observed as only one spot.

Dot blotting: The NSE level in the MUE group was significantly higher than that in the control group ($P<0.01$) (Fig. 4). The mean value in the control group was 0.84 $ng/10$ μl, and in the MUE group, it was 3.62 $ng/10$ μl. The ROC curve is shown in Fig. 5. When the cutoff value was set at 2.00 $ng/10$ μl, the diagnostic sensitivity and specificity of the assay for MUE were 96 and 93%, respectively.

DISCUSSION

In this study, a clear 2DE protein map was obtained by concentrating and desalting CSF samples from beagles using centrifugal filters. Many proteomic analyses of human CNS diseases involve sample desalting using acetone or trichloroacetic acid precipitation [1, 2, 16, 17]. In our study, desalting and concentration of small sample volumes was possible using centrifugal filters. In many cases, only

Table 2. Tryptic peptides of a common spot in patient CSFs and identified as canine neuron-specific enolase by MALDI-TOF MS analysis

| Start–End	Observed	Mr(calc)	Sequence
16–28	1380.6731	1379.7045	R.GNPTVEVDLHTAK.G
33–50	1804.9449	1803.9366	R.AAVPSGASTGIYEALELR.D
65–89	2592.3311	2591.3806	K.AVDHINTTIAPALISSGLSVVEQEK.L
106–120	1519.8061	1518.8228	K.FGANAILGVSLAVCK.A
163–179	1938.9849	1937.9743	K.LAMQEFMILPVGAESFR.D
203–228	2702.2395	2701.3082	K.DATNVGDEGGFAPNILENSEALELVK.E
240–253	1544.7983	1543.7817	K.IVIGMDVAASEFHR.D
270–285	1858.9381	1857.9261	R.YITGDQLGALYQDFVR.D
286–306	2483.088	2482.0965	R.DYPVVSIEDPFDQDDWAAWSK.F
307–326	2102.123	2101.1056	K.FTANVGIQIVGDDLTVTNPK.R
344–358	1617.806	1616.8192	K.VNQIGSVTEAIQACK.L
359–372	1583.7691	1582.7674	K.LAQENGWGVMVSHR.S
373–394	2353.1663	2352.1519	R.SGETEDTFIADLVVGLCTGQIK.T
413–422	1160.4889	1159.5357	R.IEEELGDEAR.F
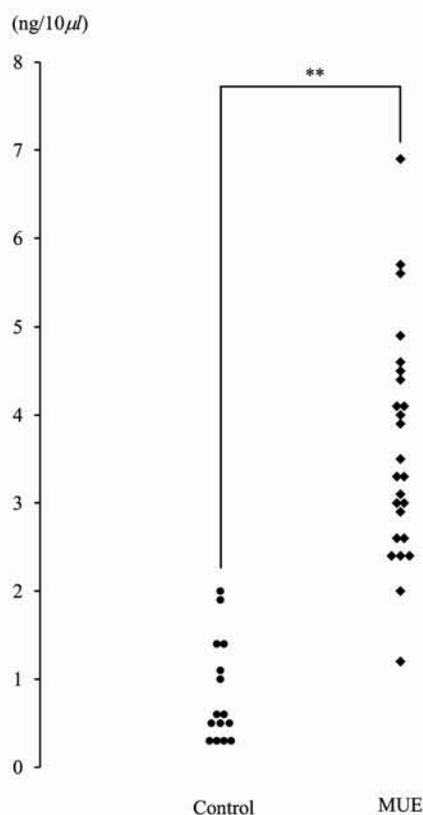
Fig. 4. Concentrations of NSE in cerebrospinal fluid of MUE subjects and in the control group. NSE concentrations of MUE subjects were significantly higher than those of the control group.
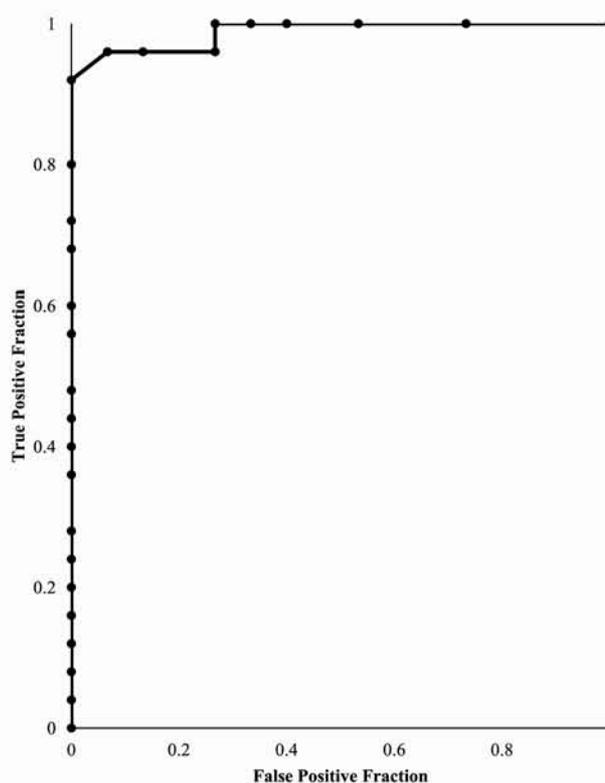

Fig. 5. The ROC curve of the NSE level in CSF. The area of under curve (AUC) was 0.9983.

100–500 μl of CSF can be collected from small animals; therefore, the use of centrifugal filters for sample desalting would be useful for proteome analyses in dogs. Because proteins of less than 3 kDa are removed with the salts using these filters, all proteins could not be analyzed in our study. However, many of the proteins in canine CSF that we identified after centrifugal filter desalting have been identified in 2DE studies in humans and other animals [2, 7, 8].

Neuron-specific enolase (NSE) is known as a biomarker of CNS disorders, for example, ischemia, head trauma, spinal cord injury and some of tumors, in humans [3, 6, 9, 12,

13, 15]. NSE is found in neurons and neuroendocrine cells. In present study, the concentration of NSE of healthy dogs was higher than that found in a previous report [14]. This is likely due to the difference in methodology and age of the control group. In humans, there is evidence that the NSE concentration in the CSF rises with age [18]. In the previous report, the dogs were 1–13 months old [14]. In the present study, the age of the healthy dogs was 2–9 years old with an average age of 4.9 ± 2.8 years old. Also, the two dogs that showed the highest NSE concentrations in the control group were 9 years old. It seems that the NSE concentration rises in canine CSF according to age, as in humans.

Though MUE dogs were generally younger than healthy dogs, NSE was elevated in the canine CSF in conjunction with the inflammation. Because NSE is usually present in a nerve cell, it is possible that NSE leaked as a result of neuronal destruction. In previous report, even GM1 gangliosidosis increased, so an increasing NSE level may not be specific in encephalitis [14]. Also, we used only beagles as a healthy dog group in this study. It will be necessary to evaluate measurement of NSE with various dog species and at different age. In humans, NSE is measured as a serum marker. We will examine the measurement of serum NSE concentration in dogs. In the present study, we only compared healthy dogs to MUE dogs, so future studies are required to measure the NSE concentrations of the CSF of dogs that have been histopathologically diagnosed with NME, GME or NLE, in addition to other inflammatory disorders, trauma, stroke and brain tumors.

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