J. Vet. Sci. (2001), **2**(1), 25–31

JOURNAL OF
Veterinary
Science

Immunohistochemical detection of Prion protein (PrP-Sc) and epidemiological study of BSE in Korea

Hye Cheong Koo, Yong Ho Park*, Byeong-Chun Lee[1], Chanhee Chae[2], Katherine I. O´Rourke[3] and Timothy V. Baszler[4]

Department of Microbiology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea
[1]Department of Theriogenology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea
[2]Department. of Pathology, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea
[3]U.S. Department Agriculture, Agricultural Research Service, Pullman, WA 99164, U.S.A.
[4]Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164, U.S.A.

Though the aetiology of transmissible spongiform encephalopathies (TSEs) remains uncertain, proteinase resistant prion protein (PrP-Sc), a converted form of the normal cellular prion protein (PrP-C), accumulates in the lysosome of cells of the nervous systems of animals with TSEs. In this study, clinical and epidemiological examinations of bovine spongiform encephalopathy (BSE) were conducted in Korea. During the investigated period, none of the cattle exhibited typical clinical signs of BSE, such as behavioral disturbances, high sensitivity, and abnormal locomotion. Immunohistochemical analysis and western immunoblotting were established to detect PrP-Sc in the brain tissue using monoclonal antibody (MAb) F89/160.1.5, produced by immunizing mice with a synthetic peptide which corresponds to bovine PrP residues 146-159, NH_2-SRPLIHFGSDYEDRC-COOH. Although some BSE-like spongiform changes were observed in bovine brains randomly collected from Korean slaughterhouses from 1996 to 1999, no PrP-Sc was detected in those brains with the established immunohistochemistry and western immunoblotting assay. Also, no positive reaction was observed in bovine brains infected with rabies. These immunohistochemical and western immunoblotting methods using MAbs, specifically reactive with conserved epitopes on ruminant PrP, can be used for postmortem diagnosis of BSE. Further, the method can be applied to antemortem and the preclinical diagnosis of ovine scrapie by detecting PrP-Sc in lymphoid tissues, such as the tonsils, third eyelid or peripheral lymph nodes.

Key words : Immunohistochemistry, Western immunoblotting, PrP-Sc, PrP-C, bovine spongiform encephalopathy

*Corresponding author
Phone: +82-31-290-2735; Fax: +82-31-295-7524
E-mail: yhp@plaza.snu.ac.kr

Introduction

Bovine spongiform encephalopathy (BSE) is one of family of diseases, the transmissible spongiform encephalopathies (TSEs) that include scrapie in sheep, a chronic wasting disease in deer, feline spongiform encephalopathy, and Creutzfeldt Jakob disease and Kuru in man [7, 25, 35, 36]. Prions are the infectious particles [4, 8, 16, 26] responsible for TSEs. They consist, at least in part, of an isoform (PrP-Sc) of the ubiquitous cellular prion protein (PrP-C) [14, 16, 24]. TSEs have a prolonged incubation period and result in chronic, progressive and ultimately fatal neurodegeneration of the CNS. TSEs provoke neither overt immune nor inflammatory responses since PrP-C and PrP-Sc are derived from the same single-copy host gene [20]. Diagnosis of TSEs has been mainly based upon clinical signs such as apprehension, hyperaesthesia and gait incoordination [9, 39, 38, 40, 43] and the microscope examination of tissues for neuropathological lesions, including vacuolation of neuronal soma and neurites (spongiform changes), and the degeneration and loss of neurones, a reactive astrocytosis and microgliosis [3, 5, 29, 31]. The purpose of this study was to establish immunohistochemistry and western immunoblotting assays with MAb F89/160.1.5 or MAb F99/97.6.1, which are specifically reactive with conserved epitopes on ruminant PrP, for the detection of PrP-Sc, and thereby to confirm TSEs in ruminants in Korea and to differentiate TSEs from other abnormalities with neuropathologic lesions displaying spongiform changes. These assays were used to investigate whether PrP-Sc is present in brain tissues of cattle with suspect clinical signs and histopathologic changes from 1996 to 1999 in Korea.

Materials and Methods

Clinical and epidemiological study of BSE in Korea

A total of 26,688 cattle, most over 2-years-old, from 811 Korean farms were examined for clinical signs of BSE. Cattle with neurologic symptoms were referred for histopathological and immunological examination, and a total of 140 bovine brains were collected from slaughterhouses in Korea between 1996 and 1999.

Monoclonal and polyclonal antibodies to PrP

Monoclonal antibody F89/160.1.5 and MAb F99/97.6.1 [21, 22, 23] were provided by Washington State University. This antibody reacts with the epitope IHFG, conserved in most ruminant species. Polyclonal mouse serum was produced by immunization with a peptide based on bovine Prion protein residues 146-159 (NH_2-SRPLIHFGS-DYEDRC-COOH), containing an added C-terminal cysteine for conjugation was synthesized [11] and conjugated to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce Chemical Company, Rockford, IL, USA) for immunogen preparation, and conjugated to BSA as the coating antigen for enzyme-linked immunosorbent assay (ELISA). Female BALB/c mice (age 6 to 10 weeks) were injected subcutaneously at 21 day intervals with 10 μg of peptide-KLH conjugate in Ribi adjuvant (RAS; RIBI ImmunoChem Research, Inc., Hamilton, MT, USA) according to the manufacturer's recommendations. Sera were screened by indirect ELISA using a recombinant sheep PrP-C in *Escherichia coli* [21, 40, 35]. Each well of the ELISA plates (Costar, Cambridge, MA, USA) was coated with 10 μg of peptide-BSA conjugate in 100 μl of 0.05 M carbonate buffer (pH 9.6), and the plates were incubated overnight at 4°C. Without blocking, 100 μl of antiserum or hybridoma supernatants were incubated for 45 min at 37°C. Bound antibody was detected with goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRPO) and *O*-phenylene-diamine dihydrochloride (Sigma, St. Louis, MO, USA). Optical density (O.D.) was determined at 490 nm. Normal serum from an unimmunized mouse or supernatants from isotype-matched MAbs of irrelevant specificity or normal tissue culture medium were used as negative controls. An O.D. value of over 0.5 was regarded as positive. The titer of the mouse antiserum following 5 immunizations was 1 : 316,228.

Source of brain tissue from sheep with scrapie and cattle from Korea

Brain tissues from naturally scrapie-affected sheep of USDA (U.S. Dept. Agriculture) origin were fixed in 10% buffered formalin, routinely processed, and sections were prepared on paraffin-embedded slides for positive controls. As a negative control, brain tissue from healthy sheep and cattle were obtained from farms in Korea. Four rabies-affected bovine brains as paraffin-embedded blocks were obtained from the National Veterinary Research and Quarantine Service in Korea. Brain stem was separated from 6 cattle with nervous clinical signs in Korean farms. A total of 140 bovine brains were collected from slaughterhouses in Korea from 1996 to 1999. After fixation in 10% buffered formalin and embedding in paraffin, blocks of brain tissue from 4 areas (frontal and caudal mesencephalon, pons, and medulla at the obex) were analyzed when available.

Hematoxylin-eosin (H&E) and amyloid staining and immunohistochemistry (IHC)

Sections of brain tissue were deparaffinized and hydrated by immersion in serial dilutions of ethyl alcohol, and were then stained with hematoxylin-eosin and alkaline congo red by conventional methods [6] for routine histopathological analysis. Additional tissue sections were immunostained for PrP-Sc using MAb F89/160.1.5 or MAb F99/97.6.1 [21, 22, 23] by blocking endogenous peroxidase with 0.3% hydrogen peroxide in methanol and autoclaving in distilled water at 121 for 40 min [10, 21, 22] to enhance the reactivity of PrP-Sc. Sections were incubated sequentially with primary MAb (RT, 1hr), biotinylated horse anti-mouse IgG secondary antibody, avidin-biotin-HRPO complex (ABC-peroxidase; Vector Labs, Burlingame, CA, USA), and a peroxidase substrate-chromogen (AEC; Dako Corp, Carpinteria, CA, USA), and then counterstained with Mayers hematoxylin. 3% normal horse serum (NHS) / 0.1 M Tris-HCl containing 0.05% Triton X-100 (TTB) was used as a diluent for primary and secondary antibodies and 0.5 M NaCl / TTB as a diluent for the ABC reagent to block non-specific tissue sites. Negative controls were prepared using brain tissue from normal sheep and cattle with no evidence of TSE and an irrelevant control MAb of the same isotype was substituted for MAb F89/160.1.5.

Western immunoblotting

To purify PrP-C and PrP-Sc by immunoprecipitation, 200 μl of a 1% brain homogenate in lysis buffer, precleared by centrifugation at 13,000 g for 15 min, was incubated for 2h at room temperature with 0.1 mg of F89/160.1.5. After incubation with an additional 50 μl of protein G-coupled agarose (Roche, Mannheim, Germany) for 2 h at room temperature, the immune complexes were centrifuged at 13,000 g for 3 min and the pellets washed according to the manufacturer's instructions [15]. Proteinase K (PK; GIBCOBRAL, Grand Island, NY, USA) digestions of immunoprecipitates were done with 20 mg/ml PK for 30 min at 37°C. Since frozen tissues of animals with TSEs could not be imported into Korea, recombinant sheep PrP was used as a positive control in western immunoblotting. PrP was analyzed in aliquots equivalent to 200 mg of starting material on 15% polyacry-

lamide minigels, and transferred to polyvinylidene difluoride membranes (NEN life science, Boston, MA, USA). The membranes were developed with 1 : 1,000 dilutions of polyclonal mouse anti-PrP, or control serum from an uninoculated mouse, goat anti-mouse IgG-HRPO, and a chemiluminescent substrate (ECL; Amersham, Buckinghamshire, England). Membranes were exposed to film (ECL Hyperfilm; Amersham) for 1-10 min.

Results

Clinical and epidemiological study of BSE in Korea

As a result of the clinical and epidemiological investigation of BSE in Korea from 1996 to 1999, a total of 162 (0.61%) of the 26,688 cattle examined were judged to be displaying neurological symptoms. During this period, the most frequently diagnosed neurological symptoms found were, 132 cases with parturient paresis (81.5%), which was followed by metabolic disease 4.3%, traumatic injuries 4.3%, and unknown causes 3.7%.

H&E and amyloid staining

The H&E staining of brain stem and mesencephalon from bovine brain obtained from Korean slaughterhouses between 1996 and 1999 occasionally showed a small number of spongiform lesions with vacuolation (Fig. 1). When cattle with clinically neurological signs were examined histopathologically, neither typical lesions of BSE, spongiform changes, nor neurofilaments were observed.

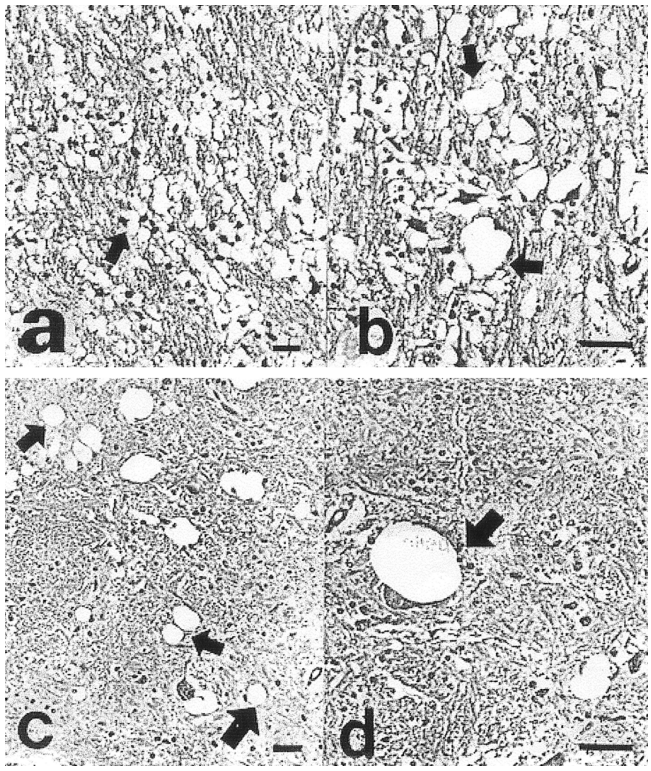

Fig. 1. Hematoxylin-eosin (H&E) staining of brain stem of cattle obtained from slaughterhouses in Korea. (a), (b); Spongiform lesion in myelins (small arrows) (c), (d); Spongiform lesion consisting of neuropil spongiosis (small arrows) and intraneuronal vacuoles (large arrows) Bar = 50 μm.

Immunohistochemistry

Brain from scrapie infected sheep was used as a positive control. Positive red PrP-Sc deposits were observed in brain tissue from naturally scrapie-affected sheep in IHC (Fig. 2). The PrP-Sc accumulation pattern was similar to that observed with polyclonal antisera to ovine or mouse PrP [18, 19] and the distribution or intensity of the immunostaining of the brain stem observed with MAb F99/97.6.1 was similar to that observed with MAb F89/160.1.5. The most abundant neuronal PrP-Sc accumulation area was the medulla at the obex and the pons, but amyloid was mostly observed in the thalamus [34] in the scrapie-affected sheep. In all areas of the brain in which vacuolation of neurons or neuropil was seen in H&E staining, PrP-Sc was detected in IHC. However, many areas containing PrP-Sc showed no vacuolation. PrP-Sc antigen accumulated within brain stem nuclei with spongiform lesions consisting of intraneuronal vacuoles and nonspongiform lesions.

PrP-Sc antigen accumulation was not observed when randomly selected cattle from slaughterhouses (Fig. 3a) or cattle with clinically neurological signs (Fig. 3b) were examined by immunohistochemistry. No immunostaining was detected in the brains of rabies-affected cattle or normal healthy sheep and cattle from farms and slaughterhouses in Korea.

Western blot analysis

Immunoprecipitation using MAb F89/160.1.5 followed by SDS-PAGE and western immunoblotting with polyclonal mouse antiserum was used to detect PrP-Sc in bovine brain tissues. Immunoprecipitated immune complexes were treated with loading buffer containing 2-mercaptoethanol (2-ME; Sigma), resolved on polyacrylamide gels and immunoblotted using the polyclonal antiserum against peptide-KLH conjugates as primary antibody. In the presence of 2- ME, only the 30 kDa monomeric recombinant PrP-C (Fig. 4, lane 1) and the PK-sensitive bovine PrP-C (Fig. 4, lane 3, 5, 7) of cattle from slaughterhouses and cattle with clinical neurologic signs was seen. Bands at 50kD and 25 kDa result from the reactivity of the secondary antibody with MAb F89/160.1.5. No bands were detected in PK-treated recombinant sheep PrP-C (Fig. 4, lane 2), bovine brain sample (Fig. 4, lane 4, 6, 8) or BSA (Fig. 4, lane 9).

Discussion

Several extensive neurohistological studies have been

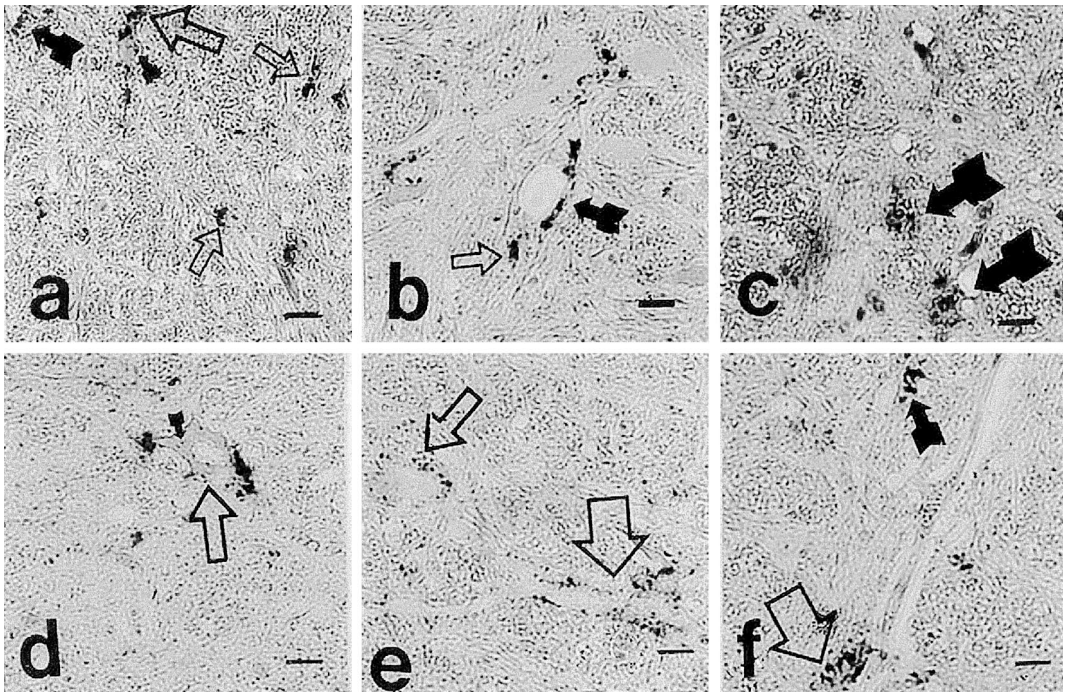

Fig 2. IHC analysis of the brain stem of scrapie-affected sheep using MAb (F89/160.1.5). ABC immunoperoxidase counterstained with Mayer's hematoxylin was used. PrP-Sc antigen accumulated (red) within a brain stem nucleus with spongiform lesions consisting of intraneuronal vacuoles and nonspongiform lesions. Immunoreactivity comprised granular and globular foci around the periphery of intraneuronal vacuoles (medium opaque arrows of a,b,f) and linear rimming around neurons (medium transparent arrows of a,d,e), plaques in the neuropil (small transparent arrows of a,b), and aggregation around glial cells with small hyperchromatic nuclei consistent with microglia (large opaque arrows of c), linear rimming around blood vessels (large transparent arrows of e,f) and punctuate granules within soma of neurons without intraneuronal vacuoles (small opaque arrows of a,d). This staining pattern was reproduced by MAb F99/97.6.1. (a)Bar = 50 μm. (b - e)Bar = 25 μm.

carried out to determine, where possible, a specific differential diagnosis for cattle with clinical neurologic signs [1, 2, 17, 42]. One of the most common findings was of encephalitis, in many cases associated with listeriosis and rabies. A number of other inflammatory conditions of unknown aetiology have been also observed. However, the greatest proportion (55-65%) of animals not confirmed as BSE had no significant neurohistological lesions [42]. A number of clinical conditions may account for at least some of these animals. Previously uncharacterized behavioural psychoses have been encountered, and cystic ovarian disease can resemble BSE. Cachexia of unknown origin can be difficult to distinguish from BSE, especially when ectoparasite trauma or stress coexist. Transient metabolic/nutritional disorders, due to abrupt alterations in nutrition or environment, can also cause genuine clinical confusion with BSE. Human factors may also contribute to the reporting of clinically normal cows as suspects, particularly an anxiety to detect cases (usually on farms with multiple previous cases) and an ignorance of the normal behaviour of cows. In summary, a miscellany of other conditions, some better characterized than others, can be confused clinically with BSE, and effective therapy is often the most appropriate means of differentiation.

Neuropathologic lesions with TSEs-like spongiform changes are also observed in other abnormalities including branched-chain-keto-aciddecarboxylase deficiency, lysosomal disorders, hepatic encephalopathy, salt intoxication, toxins (ammonium and hexachlorophene), tunicamycin, the anthelmintic chlosantel, rabies, and artifacts in tissue processing [13, 17, 33, 41]. Although histopathological examination for spongiform change has always been the principal laboratory test for the confirmation of BSE, the detection of disease-specific 'scrapie associated fibrils (SAF)' by electron microscopy [32] is an useful supporting test, particularly where tissue is autolyzed, mechanically damaged or the histopatholotical result has been equivocal. Biochemically, these SAFs appear to be composed almost entirely of the abnormal protein PrP-Sc (27-30 kDa) which is partially resistant to the proteinase enzymes used in the extraction procedure, though the normal host PrP-C (33-35 kDa) is completely denatured by proteinase enzyme digestion. The immunohistochemical demonstration of accumulated PrP in the nervous system is a diagnostic feature of the TSEs. Since most of antibodies presently available against PrP, except one [15], react with both the normal and the disease specific isoforms of the protein, protocols including autoclaving or PK digestion and controls are

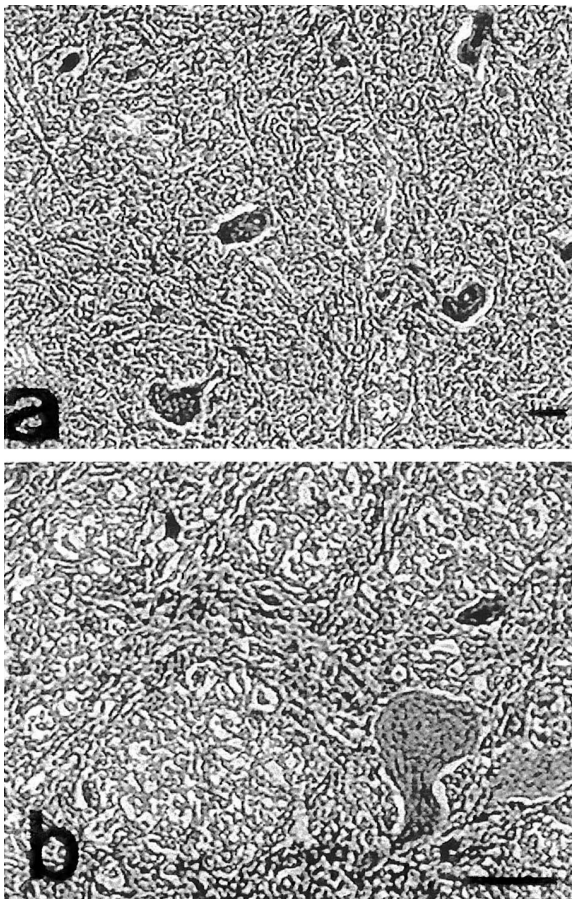

Fig. 3. Representative IHC of brain stem of slaughterhouses cattle (a) and of brain stem (b) of cattle with nervous signs. No PrP-Sc antigen accumulation was observed. Similar results were obtained with brain tissue from scrapie-affected sheep immunostained with irrelevant isotype-matched MAb. Bar = 25 μm.

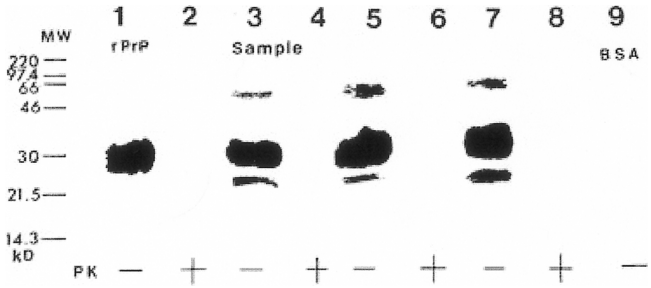

Fig 4. Result of SDS-PAGE and western immunoblotting with mouse polyclonal antisera to peptide-KLH conjugates. Immunoprecipitation of bovine PrP with MAb F89/160.1.5 showed PrP-C at 30kDa in the lane of PK-untreated recombinant sheep PrP (lane 1) and brain tissue from cattle obtained from slaughterhouses and farms (lanes 3, 5, 7). In PK-treated recombinant sheep PrP-C (lane 2), bovine brain sample (lanes 4, 6, 8) and BSA (lane 9), the 30kDa band was not observed. In lanes 3, 5, and 7, heavy chain and light chain MAb used in the immunoprecipitation reacted with secondary antibody used in western immunoblotting at 50kDa and 25kDa. Bars on the left indicate molecular size markers (in kilodaltons).

required which exclude or identify reactivity with the normal isoform. It is always necessary to establish the disease specificity of the variety of configurations and patterns of labelling produced with each antibody. Widespread particulate staining of certain gray matter neuropil is the principal form of disease-specific immunolabelling [18, 19, 34] and a knowledge of the disease-specific configuration and distribution of Pr-P-Sc immunolabelling is central to the diagnostic application of these methods. H&E staining of bovine brain stem obtained from slaughterhouses in Korea showed TSEs-like spongiform lesions, but BSE-associated fibril protein or PrP-Sc antigen accumulation was not observed in amyloid stain, IHC analysis or western immunoblotting. Therefore, these vacuolations were attributed to other abnormalities or artifacts. No PrP-Sc band in western immunoblotting was shown in the rabies-affected bovine brain or in cattle with nervous clinical signs, as observed in farms or in cattle from slaughterhouses in Korea, during the study period.

The immunohistochemistry or western immunoblotting technique described in this report is suitable for the assay of bovine brain, retina, distal ileum, deer or elk brain, and of ovine brain, spleen and lymphoid tissues, including the peripheral lymph nodes, tonsil and nictitating membrane (third eyelid, palpebra tertia) [12, 22, 23, 27, 28, 30, 43]. MAbs to a conserved site and a standard immunological assay technqiue can be useful in combination with clinical and histopathological analysis for the surveillance of TSEs in Korean livestock. The characterization of MAb, YHP99/1.5, YHP99/8.6, YHP99/8.11, YHP99/9.2, YHP99/9.5, which were produced against bovine Prion protein residues 146-159 (NH_2-SRPLIHFGSDYEDRC-COOH) in Korea, and their application for the preclinical and postmortem diagnosis of scrapie, BSE are under investigation.

Acknowledgements

Appreciation is extended to Dr. Kim, Dae-Yong, Department of Pathology, College of Veterinary Medicine, Seoul National University, Korea for histopathological advice and comments. We also thank Laurel Lafon, Sook Shin, Keun Seok Seo, Soo Jin Yang, and So Hyun Kim for technical assistance and critical discussion of this manuscript. This research was supported by the Rural Development Administration, the Ministry of Agriculture and Forest, and the Research Institute for Veterinary Science of the College of Veterinary Medicine, Seoul National University, Korea. The project was also supported in part by the Brain Korea 21 project.

References

1. **Braun, U., Kihm, U., Pusterla, N. and Schonmann, M.** Clinical examination upon suspicion of bovine spongiform encephalopathy(BSE). Schweiz. Arch. Tierheilkd. 1997, **139**, 35-41.
2. **Braun, U., Schicker, E. and Hornlimann, B.** Diagnostic reliability of clinical signs in cows with suspected bovine spongiform encephalopathy. Vet. Rec. 1998, **143**, 101-105.
3. **Brown, D. R. Schmidt, B. and Kretzschmar H.A.** A neurotoxic prion protein fragment enhances proliferation of microglia but not astrocytes in culture. Glia 1996, **18**, 59-67.
4. **Come, J. H., Fraser, P. E. and Lansbury, P. T.** A kinetic model for amyloid formation in the prion diseases: importance of seeding. Proc. Natl. Acad. Sci. USA 1993, **90**, 5959-5963.
5. **Forloni, G., Bo, R. G., Angeretti, N., Chiesa, R., Smiroldo, S., Doni, R., Ghibaudi, E., Salmona, M., Porro, M. and Verga, L.** A neruotoxic prion protein fragment induces rat astroglial proliferation and hypertrophy. Eur. J. Neurosci. 1994, **6**, 1415-1422.
6. **Fraser, H.** The pathology of natural and experimental scrapie. In : Kimberlin RH (ed) Slow virus Diseases of Animals and Man, pp 267-305. 1976.
7. **Gabizon, R. and Prusiner, S. B.** Prion liposomes. Biochem. J. 1990, **266**, 1-14.
8. **Gajdusek, D. C.** Genetic control of nucleation and polymerization of host precursors to infectious amyloids in the transmissible amyloidosis of brain. Br. Med. Bull. 1993, **49**, 913-932.
9. **Hadlow, W. J., Kennedy, R. C. and Race, R. E.** Natural infection of Suffolk sheep with scrapie virus. J. Infect. Dis. 1982, **146**, 657-664.
10. **Haritani, M., Spencer, Y. I. and Wells, F. A. H.** Hydrated autoclavable pretreatment enhancement of prion protein immunoreactivity in formalin-fixed bovine spongiform encephpalophthy-affected brain. Acta. Neuropathol. 1992, **87**, 86-90.
11. **Horiuchi, M., Yamazaki, N., Ikeda, T., Ishiguro, N. and Shinagawa, M.** A cellular form of the prion protein (PrPc) exists in many non-neuronal tissues of sheep. J. Gen. Virol. 1995, **76**, 2583-2587.
12. **Ikegami, Y., Ito, M., Isomura, J., Momotani, E., Sasak, K., Muramatsu, Y., Ishiguro, N. and Shinagawa, M.** Preclinical and clinical diagnosis of scrapie by detection of PrP protein in tissues of sheep. Vet. Rec. 1991, **128**, 271-275.
13. **Jeffrey, M. and Wilesmith, J. W.** Idiopathic brainstem neuronal chromatolysis and hippocampal sclerosis: a novel encephalopathy in clinically suspect cases of bovine spongiform encephalopathy. Vet. Rec. 1992, **131(16)**, 359-362.
14. **Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T. and Caughey, B.** Cell-free formation of protease-resistant prion protein. Nature 1994, **370**, 471-474.
15. **Korth, C., Stierli, B., Streit, P., Moser, M., Schalter, O., Fischer, R., Schulz-Schaeffer, W., Kretzschmar, H., Raeber, A., Braun, U., Ehrensperger, F., Hornemann, S., Glockshuber, R., Riek, R., Billeter, M., Wuthrich, K. and Oesch, B.** Prion (PrP-Sc)-specific epitope defined by a monoclonal antibody. Nature 1997, **390**, 74-77
16. **Laurent, M.** Autocatalytic processes in cooperative mechanisms of prion disease. FEBS Lett. 1997, **407**, 1-6.
17. **McGill, I. S. and Wells, G. A. H.** Neuropathological findings in cattle with clinically suspect but histologically unconfirmed bovine spongiform encephalopathy (BSE). J. Comp. Pathol. 1993, **108(3)**, 241-260.
18. **Miller, J. M., Jenny, A. L., Taylor, W. D., Marsh, R. F., Rubenstein, R. and Race, R. E.** Immunohistochemical detection of prion protein in sheep with scrapie. J. Vet. Diag. Invest. 1993, **5**, 309-316.
19. **Miller, J. M., Jenny, A. L., Taylor, W. D., Race, R. E., Ernst, D. R., Katz, J. B. and Race, R. E.** Detection of protein in formalin-fixed brain by hydrated autoclaving immunohistochemistry for the diagnosis of scrapie in sheep. J. Vet. Diagn. Invest. 1994, **6**, 366-368.
20. **Oesch, B., Westaway, D., Walchi, M., McKinley, M. P., Kent, S. B. H., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B. and Weissmann, C.** A cellular gene encodes scrapie PrP27-30 protein. Cell 1985, **40**, 735-745.
21. **O'Rourke, K. I., Baszler, T. V., Miller, J. M. and Knowles, D. P.** Monoclonal antibody F89/160.1.5 defines a conserved epitope on the ruminant prion protein. J. Clin. Microbiol. 1998, **36(6)**, 1750-1755.
22. **O'Rourke, K. I., Baszler, T. V., Parish, S. M. and Knowles, D. P.** Preclinical detection of PrP-scrapie in nictiating membrane lymphoid tissue of sheep. Vet. Rec. 1998, **142**, 489-491.
23. **O'Rourke, K. I., Baszler, T. V., Besser, T. E., Miller, J. M., Cutlip, R. C., Wells, G. A. H., Ryder, S. J., Parish, S. M., Hamir, A. N., Cockett, N. E., Jenny, A. and Knowles, D. P.** Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. Vet. Rec. 2000, **38(9)**, 3254-3259.
24. **Prusiner, S. B.** Novel proteinaceous infections particles cause scrapie. Science 1982, **216**, 136-144.
25. **Prusiner, S. B.** Molecular biology of prion diseases. Science 1991, **252**, 1515-1522.
26. **Prusiner, S. B.** Human prion diseases and neurodegeneration. Curr. Top. Microbiol. Immunol. 1996, **207**, 1-17.
27. **Race, R., Ernst, D., Jenny, A., Taylor, W., Sutton, D. and Caughey, B.** Diagnostic implications of detection of proteinase K-resistant protein in spleen, lymph nodes, and brain of sheep. Am. J. Vet . Res. 1992, **53(6)**, 883-889.
28. **Roles, S., Vanopdenbosch, E., Langeveld, J. P. M. and Schreuder, B. E. C.** Immunohistochemical evaluation of tonsillar tissue for preclinical screening of scrapie based on surveillance in Belgium. Vet. Rec. 1999, **145**, 524-525.
29. **Rubenstein, R., Hui, D., Race, R., Hu, W., Scalici, C., Papini, M., Kascsak, R. and Carp, R.** Replication of scrapie stains *in vitro* and their influence on neuronal functions. Ann. N. Y. Acad .Sci. 1994, **724**, 332-337.
30. **Schreuder, B. E. C., van Keulen, L. J. M., Vromans, M. E. W., Langeveld, J. P. M. and Smits, M. A.** Tonsillar biopsy and PrP^{Sc} detection in the preclinical diagnosis of scrapie. Vet. Rec. 1998, **142**, 564-568.

31. **Selvaggini, C., De Gioia, S., Cantu, L., Ghibaudi, E., Diomede, L., Passerini, F., Forloni, G., Bugiani, O., Tagliavini, F. and Salmona, M.** Molecular characteristics of a protease-resistant, amyloidogenic and neurotoxic peptide homologous to residues 106-126 of the prion protein. Biochem. Biophys. Res. Commun. 1993, **194**, 1380-1389.
32. **Stack, M. J., Keyes, P. and Scott, A. C.** The diagnosis of bovine spongiform encephalopathy and scrapie by the detection of fibrils and the abnormal protein isoform. In: H. F. Baker and R. M. Ridley (ed) Prion diseases. Human Press, Totowa, NJ, pp 85-103, 1996.
33. **Summers, B. A., Cummings, J. F. and de Lahunta, A.** Principles of neuropathology. In : Summers, BA, J F Cummings, and A de Lahunta(ed) Veterinary neuropathology. Mosby-Year Book Inc, St. Louis, MO, 1995.
34. **van Keulen, L. J. M., Schreuder, B. E. C., Meloen, R. H., Poelen-van den Berg, M., Mooij-Harkes, G., Vromans, M. E. W. and Jangveld, J. P. M.** Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. Vet. Pathol. 1995, **32**, 299-308.
35. **Weismann, C.** The prion's progress. Nature 1991, **349**, 569-570.
36. **Weismann, C.** A 'unified theory' of prion propagation. Nature 1991, **352**, 679-683.
37. **Wells, G. A. H., Hawkins, S. A. C., Green, R. B., Austin, A. R., Dexter, I., Spencer, Y. I., Chaplin, M. J., Stack, M. J. and Dawson, M.** Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update .Vet. Rec. 1998, **142**, 103-106.
38. **Wells, G. A. H. and McGiill, I.S.** Recently described scrapie-like encephalopathies of animals: case definitions. Res.Vet. Sci. 1992, **53**, 1-10.
39. **Wells, G. A. H., Scott, A. C., Johnson, C. T., Gunning, R. D., Hancock, R. D., Jeffrey, M., Dawson, M. and Bradley, R.** A novel progressive spongiform encephalopathy in cattle. Vet. Rec. 1987, **121**, 419-420.
40. **Wells, G. A. H. and Simmons, M. M.** The essential lesion profile of bovine spongiform encephalophthy (BSE) in cattle is unaffected by breed or route of infection. Neuropathol. Appl. Neurobiol. 1996, **22**, 453.
41. **Wells, G. A. H. and Wells, M.** Neuropil vacuolation in brain : a reproducible histological precessing artefact. J. Comp. Pathol. 1989, **101(4)**, 355-362.
42. **Wilesmith, J. W., Wells G. A. H., Cranwell, M. P. and Ryan, J. B. M.** Bovine spongiform encephalopathy: epidemiological studies. Vet. Rec. 1988, **123**, 638-644.
43. **Williams, E. S. and Young, S.** Chronic wasting disease of captive mule deer: spongiform encephalopathy. J. Wildl. Dis. 1980, **16**, 89-98.