J Vet Diagn Invest 18:106–109 (2006)

Comparison of three monoclonal antibodies for use in immunohistochemical detection of bovine spongiform encephalopathy protease-resistant prion protein

Francisco J. Salguero,[1] Fayna Díaz-San Segundo, Alejandro Brun, María J. Cano, Juan M. Torres

Abstract. Confirmatory diagnosis of prion diseases in humans and animals relies on the histopathological examination and immunodetection of the protease-resistant isoform of prion protein (PrP^{res}). The generation of novel PrP-specific monoclonal antibodies (MAbs) has greatly improved diagnostic methodology and basic research on prion diseases as well. In this study, the performance of 3 different PrP-specific MAbs in recognizing brain PrP^{res} deposits from cows affected with bovine spongiform encephalopathy (BSE) was compared by using a standard immunohistochemical technique under different pretreatment conditions. All antibodies showed similar reactivity after denaturing treatment. However, greater differences were found among them after proteinase K treatment, even in the absence of a denaturing step. In fact, 1 MAb (2A11) was able to react with PrP^{res} deposits in the absence of a denaturing step, yielding the strongest signal and confirming the usefulness of MAb 2A11 in immunohistochemistry for the diagnosis of BSE.

Key words: Bovine spongiform encephalopathy; diagnosis; immunohistochemistry; monoclonal antibody; prion.

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, belong to a new class of infectious disorders affecting both humans and animals.[20] Among prion diseases, bovine spongiform encephalopathy (BSE) has become a serious public health concern after a link was established between the emergence of a new variant of Creutzfeld-Jakob disease in humans and dietary exposure to BSE-contaminated food.[4] From the appearance of the first BSE case to the adoption of control and surveillance measures, the epidemic reached a peak of more than 170,000 confirmed cases, mostly in the United Kingdom and other European countries.[1] Because of the adoption of rapid postmortem immunoassays, the risk of beef and other products from BSE-infected cattle entering into the human food chain has been considerably reduced. To date, all validated assays rely on immunological detection of prion protein (PrP) by using specific antibodies.[2]

Immunohistochemical staining of the protease-resistant isoform of PrP (PrP^{res}) in brain-tissue sections and Western blot analysis of brain tissue are well-established techniques for the confirmatory diagnosis of TSEs.[15,21,22] The accumulation of a PrP^{res} in the central nervous system, together with lymphoid tissues in sheep scrapie, is the key pathological feature of TSEs, and, according to the prion theory, this abnormal isoform is the infectious agent.[20] Although some antibodies have been recently shown to discriminate between normal and pathological isoforms,[6,19] there are no PrP^{res}-specific antibodies for immunohistochemistry.[21] The specificity and sensitivity of PrP^{res} detection by immunohistochemistry are greatly affected by the methodology and the PrP antibody used.[11] In this study, 3 different monoclonal antibodies (MAbs) were compared for use in immunohistochemical staining of brainstem samples from BSE-affected cattle under different conditions. Monoclonal antibodies 6H4[a] and L42[b] were purchased, whereas MAb 2A11 was previously generated in the author's laboratory.[5] All 3 MAbs were of IgG1 isotype, kappa light chain, and had been generated by immunization of PrP knockout mice with recombinant full-length bovine PrP as immunogen. The epitopes recognized by all 3 MAbs are located between amino acid residues 150 and 170 of the PrP (Fig. 1).

Samples from 10 naturally BSE-affected cows slaughtered during the clinical stage of the disease were used. Brainstem samples containing obex and pontine area were fixed in 10% buffered formalin for 2 weeks. For histopathological and immunohistochemical examinations, the fixed tissue samples were cut into blocks (3–5-mm thick), immersed in 98–100% formic acid[c] for 1 hour to reduce infectivity,[3] washed in water for 10–15 minutes, dehydrated, and embedded in paraffin wax according to standard histopathological methods. Sections (3 μm) were placed on slides treated with 3-triethoxysilyl propilamine[c] and dried for 24 hours at 37°C. The sections were dewaxed by standard procedures and stained with hematoxylin and eosin or used for immunohistochemistry. The following pretreatments or combinations were used for immu-

From the Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera Algete-El Casar s/n. Valdeolmos, 28130 Madrid, Spain.

[1]Corresponding Author: Francisco J. Salguero Bodes, Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera Algete-El Casar s/n. Valdeolmos, 28130 Madrid, Spain. salguero@inia.es

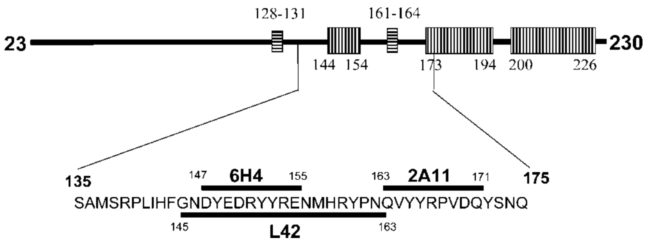

Figure 1. Epitope location on the prion protein (PrP) sequence (▤, α-helix; ▥, β-sheet) for 2A11, 6H4, and L42 monoclonal antibodies. Codon numeration corresponds to ovine PrP sequence.

Table 1. Immunolabeling of protease-resistant isoform of prion protein, graded from negative (−) to strong positive (+++), by using monoclonal antibodies (MAbs) 6H4, 2A11, and L42 and different pretreatments.*

MAB/ pretreatment	Untreated	PK	GdSCN	GdSCN + PK
6H4	−	−	++	++
2A11	++	+++	++	+++
L42	−	−	++	++

* PK = proteinase K pretreatment; GdSCN = guanidine isothiocyanate pretreatment.

nolabeling: 1) Formic acid: sections were immersed a second time in 98–100% formic acid for 15 minutes followed by rinsing in tap water for 10 minutes and washing in phosphate-buffered saline (PBS) (pH 7.2). This pretreatment was performed for all slides to ensure the reduction of infectivity.[3] 2) Proteinase K: sections were immersed in tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 20 μg/ml of proteinase K[d] for 15 minutes at 37°C followed by thorough TBS washing. 3) Guanidine isothiocyanate: sections were immersed in 4 M guanidine isothiocyanate[e] for 2 hours at 4°C. 4) Guanidine isothiocyanate treatment followed by proteinase K digestion as above. Untreated sections were also used for immunolabeling.

Sections were incubated overnight at 4°C in a humid chamber with each primary antibody. Antibodies were diluted (1:400) in PBS containing normal goat serum 10%. The immunolabeling of sections was visualized by the avidin–biotin–peroxidase complex method Vectastain Elite Kit[f] and 3,3′-diaminobenzidine tetrahydrochloride[e] as a chromogen, according to the manufacturers' instructions. The slides were counterstained with Mayer hematoxylin for 1 minute, dehydrated, and routinely mounted. Specific primary antibody was replaced by PBS or nonimmune mouse serum in tissue sections used as negative controls.

Histopathological findings in BSE-infected animals consisted of spongiosis and neuronal vacuolation in different areas of the brainstem. These findings were consistent with BSE. Deposition of PrP^{res} was detected in the medulla oblongata and pontine area of BSE-affected cows with all 3 MAbs (Fig. 2). Immunostaining was concentrated within specific brain nuclei such as tractus spinalis nucleus and dorsal motor nucleus of the vagus nerve. Positive reaction was observed as granular labeling of the gray mater neuropil as well as granular and spot-like intracellular and extracellular labeling. The majority of PrP^{res} immunoreactivity consisted of aggregates adjacent to or surrounding glial cell nuclei and punctate immunostaining within or around the periphery of the neuronal perikarya.

Table 1 summarizes the immunolabeling results. The MAb 2A11 did not need denaturing pretreatment with guanidine isothiocyanate to detect PrP^{res} reactivity, but proteinase K treatment was particularly effective in enhancing PrP^{res} immunolabeling (Fig. 2). In contrast, MAbs 6H4 and L42 did not show any positive immunostaining when no pretreatment was used. All 3 antibodies showed very similar results with the combination of formic acid and guanidine isothiocyanate. In contrast to what was observed for MAb 2A11, no differences in the immunoreactivity of 6H4 and L42 were observed after the proteinase K and the guanidine isothiocyanate treatments. Samples from healthy animals did not show any PrP^{res}-like immunoreaction in any area of the brainstem. Sections incubated with PBS or nonimmune mouse serum did not produce any staining.

Immunohistochemical staining is widely used for the diagnosis and pathogenesis studies of TSEs.[9,11–13,15–17,22] It is also a very useful technique for autolyzed tissues, which may not be suitable for routine histopathology.[8,18] An adequate number of antibodies have been developed and tested for use in immunohistochemical detection.[11,14]

The normal isoform of PrP (PrP^{C}) is a very labile protein that can be preserved only with special and careful methods of fixation and embedding.[10] However, PrP^{res} is much more stable and remains immunoreactive after routine fixation in 10% formalin and paraffin embedding.[10] The principal function of antigen-retrieval methods is to unmask antigens after fixation. Enzymatic digestion with proteinase K destroys PrP^{C}; formic acid, guanidine isothiocyanate, and autoclaving may also destroy the instable PrP^{C} but preserve the detection of PrP^{res}.[7]

In the present study, the efficacy of antigen-retrieval methods in the immunohistochemical diagnosis of BSE by using 3 MAbs was evaluated. The procedure included immersion of the brain-tissue block in formic acid to inactivate infectivity.[3] The MAbs, unlike polyclonal antibodies,[11] did not produce any nonspecific background staining. The major difference among the 3 MAbs tested was that the immunoreactivity of MAb

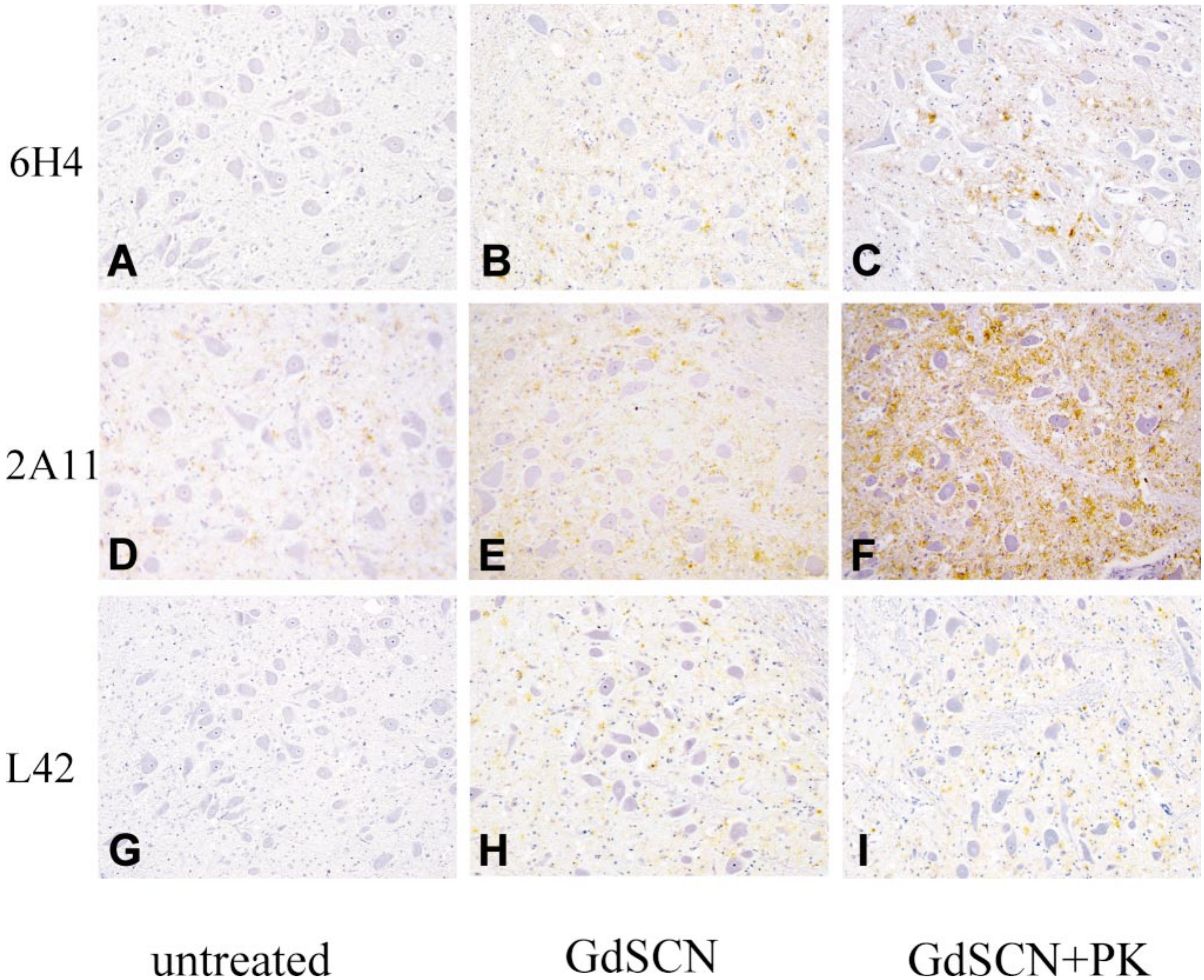

Figure 2. Immunohistochemical comparison Medulla oblongata, dorsal motor nucleus of the vagus nerve. Immunolabeling of the protease-resistant isoform of prion protein with monoclonal antibodies 6H4 (**A**, **B**, **C**), 2A11 (**D**, **E**, **F**), and L42 (**G**, **H**, **I**) without pretreatment (**A**, **D**, **G**), treated with guanidine isothiocyanate (**B**, **E**, **H**), or treated with guanidine isothiocyanate and proteinase K (**C**, **F**, **I**). ABC, 200×.

2A11 did not require an initial denaturation step with guanidine isothiocyanate. However, proteinase K enhanced specific immunoreaction. Increased immunoreactivity of the MAb 2A11 after proteinase K treatment has also been observed by Western blot.[5] The particular behavior of MAb 2A11 may be explained by optimal exposure of the PrP-specific epitope after proteinase K digestion of PrP^{res} aggregates. In contrast, MAbs 6H4 and L42 showed no increased reactivity after proteinase K treatment and needed guanidine isothiocyanate treatment to obtain positive results.

The results herein indicate that MAb 2A11 yielded the strongest signal in all cases. As expected,[5] only MAb 2A11 immunoreactivity was greatly enhanced after treatment with proteinase K. These results confirm the usefulness of MAb 2A11 in immunohistochemistry for the diagnosis of BSE.

Acknowledgements. Veterinary Laboratories Agency (UK) kindly provided the BSE naturally affected samples. This study was supported by Spanish National Grant (SC00–055) from INIA.

Sources and manufacturers

a. Prionics AG, Schlieren, Switzerland.
b. RIDA-Biopharm, Darmstadt, Germany.
c. Merck, Darmstadt, Germany.
d. Roche Diagnostics Corporation Inc., Indianapolis, IN.
e. Sigma Chemical Company, Poole, Dorset, UK.
f. Vector, Burlingame, CA.

References

1. Anonymous: 1996, World Health Organization consultation on public health issues related to bovine spongiform encephalopathy and the emergence of a new variant of Creutzfeldt-Jakob disease. Morb Mortal Wkly Rep 45:295–303.

2. Bozzetta E, Acutis PL, Martucci F, et al.: 2004, Evaluation of rapid tests for the diagnosis of transmissible spongiform encephalopathies in sheep and goats. Acta Neuropathol (Berl) 107: 559–562.
3. Brown P, Wolff A, Gajdusek DC: 1990, A simple and effective method for inactivating virus infectivity in formalin-fixed tissue samples from patients with Creutzfeldt-Jakob disease. Neurology 40:887–890.
4. Bruce ME, Will RG, Ironside JW, et al.: 1997, Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature 389:498–501.
5. Brun A, Castilla J, Ramirez MA, et al.: 2004, Proteinase K enhanced immunoreactivity of the prion protein-specific monoclonal antibody 2A11. Neurosci Res 48:75–83.
6. Curin Serbec V, Bresjanac M, Popovic M, et al.: 2004, Monoclonal antibody against a peptide of human prion protein discriminates between Creutzfeldt-Jacob's disease-affected and normal brain tissue. J Biol Chem 279:3694–3698.
7. DeArmond SJ, Mobley WC, DeMott DL, et al.: 1987, Changes in the localization of brain prion proteins during scrapie infection. Neurology 37:1271–1280.
8. Debeer SO, Baron TG, Bencsik AA: 2001, Immunohistochemistry of PrPsc within bovine spongiform encephalopathy brain samples with graded autolysis. J Histochem Cytochem 49: 1519–1524.
9. Foster JD, Wilson M, Hunter N: 1996, Immunolocalisation of the prion protein (PrP) in the brains of sheep with scrapie. Vet Rec 139:512–515.
10. Gonzalo-Pascual I, Cuadrado-Corrales N: 2000 Técnicas de inmunohistoquímica en las enfermedades por priones. Rev Neurol 31:156–159.
11. Hardt M, Baron T, Groschup MH: 2000, A comparative study of immunohistochemical methods for detecting abnormal prion protein with monoclonal and polyclonal antibodies. J Comp Pathol 122:43–53.
12. Haritani M, Spencer YI, Wells GA: 1994, Hydrated autoclave pretreatment enhancement of prion protein immunoreactivity in formalin-fixed bovine spongiform encephalopathy-affected brain. Acta Neuropathol 87:86–90.
13. Jeffrey M, Goodsir CM, Holliman A, et al.: 1998, Determination of the frequency and distribution of vascular and parenchymal amyloid with polyclonal and N-terminal-specific PrP antibodies in scrapie-affected sheep and mice. Vet Rec 142:534–537.
14. Kovacs GG, Head MW, Hegyi I, et al.: 2002, Immunohistochemistry for the prion protein: comparison of different monoclonal antibodies in human prion disease subtypes. Brain Pathol 12:1–11.
15. McBride PA, Bruce ME, Fraser H: 1988, Immunostaining of scrapie cerebral amyloid plaques with antisera raised to scrapie-associated fibrils (SAF). Neuropathol Appl Neurobiol 14:325–336.
16. Miller JM, Jenny AL, Taylor WD, et al.: 1993, Immunohistochemical detection of prion protein in sheep with scrapie. J Vet Diagn Invest 5:309–316.
17. Miller JM, Jenny AL, Taylor WD, et al.: 1994, Detection of prion protein in formalin-fixed brain by hydrated autoclaving immunohistochemistry for the diagnosis of scrapie in sheep. J Vet Diagn Invest 6:366–368.
18. Monleon E, Monzon M, Hortells P, et al.: 2003, Detection of PrP(sc) in samples presenting a very advanced degree of autolysis (BSE liquid state) by immunocytochemistry. J Histochem Cytochem 51:15–18.
19. Paramithiotis E, Pinard M, Lawton T, et al.: 2003, A prion protein epitope selective for the pathologically misfolded conformation. Nat Med 9:893–899.
20. Prusiner SB: 1991, Molecular biology of prion diseases. Science 252:1515–1522.
21. Ryder SJ, Spencer YI, Bellerby PJ, March SA: 2001, Immunohistochemical detection of PrP in the medulla oblongata of sheep: the spectrum of staining in normal and scrapie-affected sheep. Vet Rec 148:7–13.
22. van Keulen LJ, Schreuder BE, Meloen RH, et al.: 1995, Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. Vet Pathol 32:299–308.