Jpn. J. Infect. Dis., 64, 81-84, 2011

Short Communication

Atypical L-Type Bovine Spongiform Encephalopathy (L-BSE) Transmission to Cynomolgus Macaques, a Non-Human Primate

Fumiko Ono, Naomi Tase[1], Asuka Kurosawa[3], Akio Hiyaoka, Atsushi Ohyama, Yukio Tezuka, Naomi Wada[2], Yuko Sato[3], Minoru Tobiume[3], Ken'ichi Hagiwara[4], Yoshio Yamakawa[4]*, Keiji Terao[1], and Tetsutaro Sata[3]

The Corporation for Production and Research of Laboratory Primates, Tsukuba 305-0843;
[1]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba 305-0843;
[2]Department of Veterinary Medicine, Yamaguchi University, Yamaguchi 753-8515; and
[3]Department of Pathology and [4]Department of Biochemistry and Cell Biology,
National Institute of Infectious Diseases, Tokyo 162-8640, Japan

(Received December 9, 2010. Accepted December 22, 2010)

SUMMARY: A low molecular weight type of atypical bovine spongiform encephalopathy (L-BSE) was transmitted to two cynomolgus macaques by intracerebral inoculation of a brain homogenate of cattle with atypical BSE detected in Japan. They developed neurological signs and symptoms at 19 or 20 months post-inoculation and were euthanized 6 months after the onset of total paralysis. Both the incubation period and duration of the disease were shorter than those for experimental transmission of classical BSE (C-BSE) into macaques. Although the clinical manifestations, such as tremor, myoclonic jerking, and paralysis, were similar to those induced upon C-BSE transmission, no premonitory symptoms, such as hyperekplexia and depression, were evident. Most of the abnormal prion protein (PrP^{Sc}) was confined to the tissues of the central nervous system, as determined by immunohistochemistry and Western blotting. The PrP^{Sc} glycoform that accumulated in the monkey brain showed a similar profile to that of L-BSE and consistent with that in the cattle brain used as the inoculant. PrP^{Sc} staining in the cerebral cortex showed a diffuse synaptic pattern by immunohistochemistry, whereas it accumulated as fine and coarse granules and/or small plaques in the cerebellar cortex and brain stem. Severe spongiosis spread widely in the cerebral cortex, whereas florid plaques, a hallmark of variant Creutzfeldt-Jakob disease in humans, were observed in macaques inoculated with C-BSE but not in those inoculated with L-BSE.

Bovine spongiform encephalopathy (BSE) is a fatal transmissible neurodegenerative disorder of cattle caused by the BSE prion. This disease first emerged among cattle in the United Kingdom in 1987 (1) and subsequently spread throughout Europe, Japan, and North America within the next decade (2,3). Based on similarities in disease phenotype, brain pathology, brain lesion profile, and the glycoform profile of the proteinase-resistant core of prion protein (PrP^{Sc}), it was initially believed that the disease was caused by transmission of a single prion strain conferring classical BSE (C-BSE) (4–7). However, two types of BSE, with distinct biochemical and pathological characteristics from those of C-BSE, have been detected in the European Union (EU), Japan, and the United States (8–14). These atypical BSE types have been classified as H- and L-BSE (15) in the light of the high and low molecular mass fragments of the non-glycosylated PrP molecule in the proteinase K resistant core of PrP^{Sc}. In addition, the L-type BSE prion is distinguishable by its high content of monoglycosylated molecules in the core. To date, 27 cases of L-BSE and 24 cases of H-BSE have been reported worldwide (16), thus meaning that the prevalence of atypical BSE is considerably lower than that of C-BSE. However, recent studies showed that L-BSE is easily transmissible to transgenic mice expressing human (17,18) or bovine (19,20) prion protein, as well as to non-human primates (21), with shorter incubation periods than for the transmission of C-BSE to these animals. The virulent nature of L-BSE has stimulated new concern for human public health since the transmission of C-BSE to humans resulted in variant Creutzfeldt-Jakob disease (vCJD) (4–7), a new emergent prion disease.

Since September 2001, 36 BSE cattle have been found in Japan after blanket BSE testing of approximately 10 million cattle (22) by screening and active surveillance programs conducted by the Ministry of Health, Labour and Welfare and the Ministry of Agriculture, Forestry and Fisheries (23). Two cattle, slaughtered at abattoirs, were identified as having atypical BSE by confirmatory examinations involving Western blotting (WB) and/or immunohistochemistry (IHC). Both these cases were classified as L-BSE on the basis of their glycoform profiles. The first L-BSE case was reported in a young Holstein steer (23 months old; BSE/JP8) (12), whereas the second case was reported in an old Japanese meat cow (196 months old; BSE/JP24) (13). Transmission of the first L-BSE case to bovinized mouse (transgenic

*Corresponding author: Mailing address: Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2127, Fax: +81-3-5285-1157, E-mail: yamakawa@nih.go.jp

mice over-expressing bovine PrP) was unsuccessful, probably due to the extremely low amount of PrP^{Sc} and the limited sample size (24). In contrast, the second L-BSE case was readily transmitted to both bovinized mice (25) and bovine (20,26), with shorter incubation periods than those required for the transmission of C-BSE to these animals. The glycoform profile of PrP^{Sc} propagated in the recipient animals was similar to that accumulated in the brain of the donor cattle (BSE/JP24) (20,25,26). To date, the biochemical and histopathological characteristics of the JP24 case analyzed with bovinized mice and bovine have been reported to be similar to those reported for the bovine amyloidotic spongiform encephalopathy (BASE) case, a representative L-BSE originally identified and characterized in Italy in 2003 (8,18,19). In this study, we inoculated a brain homogenate of BSE/JP24 into cynomolgus macaques to investigate disease manifestation and the characteristics of L-BSE in primates in comparison with C-BSE.

Two macaques simultaneously developed neurological signs and symptoms 19–20 months post-inoculation (mpi) with the brain homogenate of BSE/JP24. The monkeys entered the terminal stage of the disease (total paralysis) at 24–25 mpi. Both the onset and duration of the disease were shorter than those reported for the transmission of C-BSE to macaques by us and other groups (27,28). The clinical manifestations such as tremor, myoclonic jerking, and paralysis were similar to those observed during the transmission of C-BSE to macaques, whereas the premonitory abnormal behaviors, such as hyperekplexia and depression, seen upon transmission of C-BSE to macaques were not evident (27).

Histopathological analysis and IHC, performed as described previously (29), showed that severe spongiform changes and the accumulation of PrP^{Sc} with various patterns were detectable in the brains of both monkeys (Fig. 1). Vacuolization was profound throughout the cerebral cortex, from the frontal to the occipital lobes (Fig. 1a). Likewise, synaptic-type PrP^{Sc} precipitation (30) was observed in the whole cerebral cortex and basal ganglia by IHC (Figs. 1b and c). Dense precipitates and plaques of PrP^{Sc}, which had been observed in cattle (JP24) brain (13), were not detected in the cerebrum of the monkeys. PrP^{Sc}, in the form of small plaques or coarse granules, was, however, detected in the molecular layer of the cerebellum (Fig. 1e). Despite the severe spongiosis in the cerebral cortex, florid plaques, which are large PrP^{Sc} plaques surrounded by vacuoles, a hallmark of vCJD (4–7,30) and C-BSE transmission to macaques (27,28), were not observed. The histopathology of the brain was therefore similar to that reported for the brain of L-BSE (BASE)-transmitted macaques (21).

Figure 2 shows the results of WB analysis of PrP^{Sc} in the brain and peripheral nerves (dorsal root ganglia). Despite the region-specific morphology of PrP^{Sc} deposition, the proportions of the di/mono/non-glycosylated forms of PrP^{Sc} propagated in various regions of the

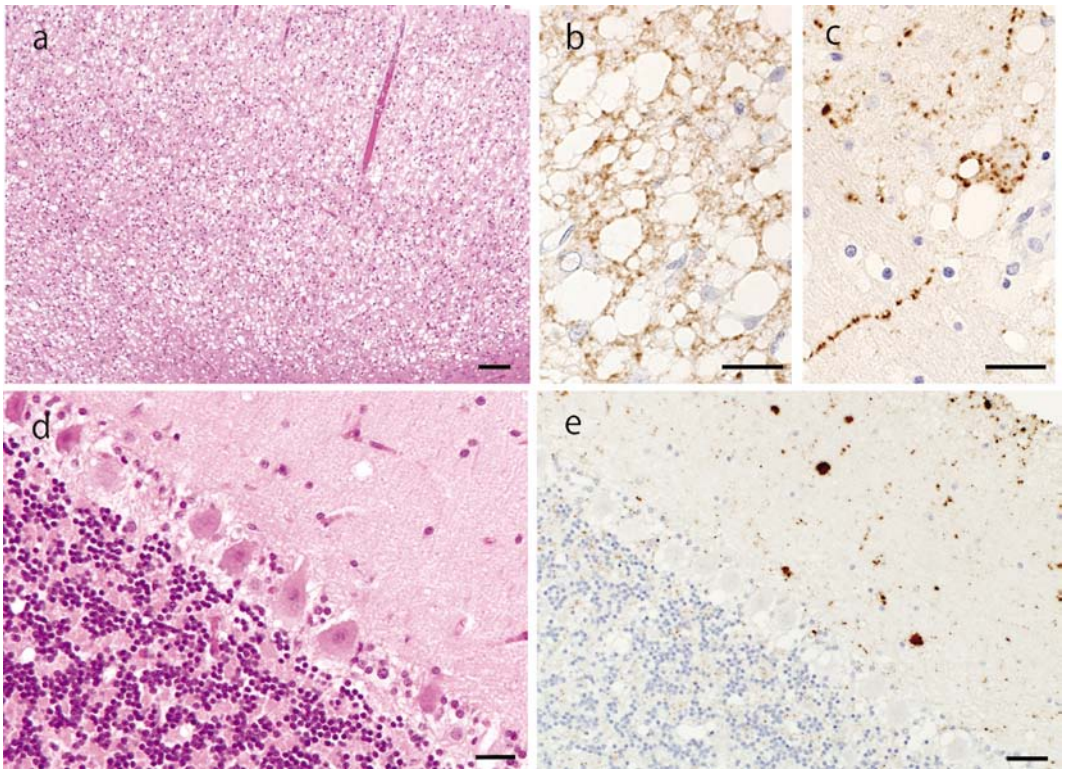

Fig. 1. Histopathology (HE) and PrP^{Sc} immunostaining of monkey brain. HE-staining of brain sections corresponding to the cortex of the cerebrum (a) and the cerebellum (d); PrP^{Sc} immunostaining of the cerebral cortex (b) and basal ganglia (c) and the cerebellum (e); a consecutive section of (d). Bar = 100 μm (a); 20 mm (b, c, d, and e). HE- and PrP^{Sc} immunostaining were performed as described previously (29). Anti-prion protein antibody T4, a rabbit polyclonal antibody raised against the synthetic peptide correspond to codons 211–239 of bovine prion protein (38), was used as the primary antibody. The CSA II amplification system (Biotin-free Catalyzed Amplification System; Dako, Kyoto, Japan) was used to enhance signal intensity instead of the Envision + immuno-enhancing system.

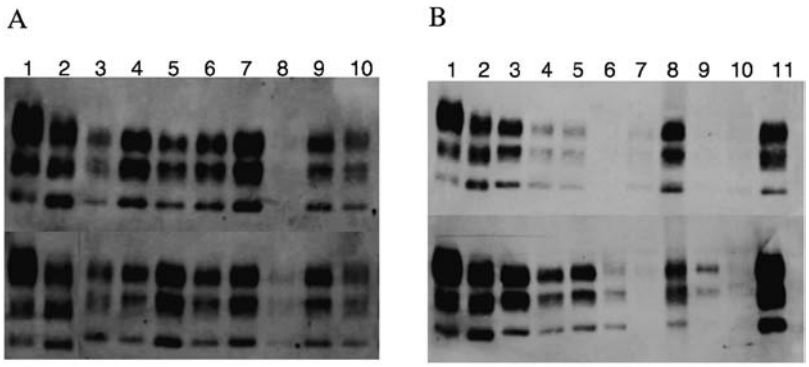

Fig. 2. PrP^{Sc} accumulated in the brain (A) and peripheral nerves (B) of euthanized monkeys. Upper and lower panels represent the brains of different monkeys, #14 and #15, respectively. (A) Lane 1, PrP^{Sc} of C-BSE (25 μg tissue equivalent); lane 2, PrP^{Sc} of L-BSE (50 μg equivalent); lanes 3–10, PrP^{Sc} accumulated in the brain of a monkey (10 μg brain tissue equivalent): lane 3, frontal lobe; lane 4, temporal lobe; lane 5, parietal lobe; lane 6, occipital lobe; lane 7, thalamus; lane 8, mid brain/medula oblongata; lane 9, cerebellum; lane 10, olfactory bulb. (B) Lanes 1–2, PrP^{Sc} of C-, L-BSE (25 and 50 μg brain equivalent); lanes 3–6, spinal cord of cervical thoracic and lumber regions (200 μg tissue equivalent): lane 6, dorsal root ganglia (1 mg tissue equivalent); lane 7, optic nerve (1 mg tissue equivalent); lane 8, retina (1 mg tissue equivalent); lane 9, trigeminal nerve (1 mg tissue equivalent); lane 10, sciatic nerve (5 mg tissue equivalent); lane 11, olfactory bulb (20 μg tissue equivalent). All techniques used in this analysis, including preparation of tissue homogenates, proteinase K treatment, poly-acrylamide gel electrophoresis on 12% gels (NuPAGE; Invitrogen, Carlsbad, Calif., USA) and transfer of protein onto PVDF membrane, were performed as described previously (27,29). Anti-prion protein antibody 6H4 (mouse monoclonal; Roche Applied Science, Basel, Switzerland) and peroxidase-labeled anti-mouse IgG antibody $(Fab)_2$, were used as the primary and secondary antibodies, respectively. Immunoreactive protein on the blot was reacted with ECLplus chemiluminescent reagent and signals were recorded and processed on a Lumino-image analyzer LAS-3000 mini (Fuji film, Tokyo, Japan). PrP^{Sc} of C-BSE and L-BSE were prepared from cattle brain by the same method and electrophoresed as in-house references to evaluate the whole process.

brain were similar to each other and to those observed in the original JP24 cattle (Fig. 2A). Although it appeared that the non-glycosylated PrP^{Sc} accumulated in the monkey tissue showed a slight upper-shift of its electrophoretic mobility towards that of C-BSE in the present WB analysis (for example, Fig. 2A lower panel), it is not known whether this minor shift was significant as the non-glycosylated PrP^{Sc} in the BSE/JP24 cattle originally had a mobility similar to, or only slightly different from, that of the C-BSE case (13,25). Further transmission experiments and a more accurate WB analysis are therefore needed to confirm such subtle differences in the non-glycosylated molecule of BSE prions after transmission to macaques. Both IHC and WB successfully detected PrP^{Sc} in retina, trigeminal ganglia, and dorsal root ganglia (Fig. 2B). PrP^{Sc} was barely detected in the sciatic nerve of monkey #15 by WB (Fig. 2B lowere panel lane 10). However, the amount of PrP^{Sc} in the sciatic nerve was estimated to be less than 1/1,000 of the cerebral PrP^{Sc}. Deposition of PrP^{Sc} in lymphoid tissues (spleen and tonsils) or lymph nodes (inguinal, axillary, submandibular, deep cervical, mesenteric, subiliac, and hilar lymph nodes) was not detected by IHC or WB. Considering the sensitivity of WB analysis and the amount of tissue (5 mg/lane) used in this experiment, the amount of PrP^{Sc} in lymphoid tissues must therefore be lower than 1/5,000 of that in the brain.

The results of an enzyme-linked immunosorbent assay for bovine PrP^{Sc} (BSE TeSeE; BioRad, Mornes-la-Coquette, France) suggested that the amount of PrP^{Sc} in the inoculum used in this study was as low as 1/5 than in a brain homogenate of C-BSE (JP6; $10^{5.2}$ LD_{50}/g using bovinized mice; see ref. 24) previously used for the transmission of C-BSE to macaques (27). Despite the low concentration of PrP^{Sc}, both the incubation period and the duration of the disease were approximately 2/3 shorter than those required for the transmission of C-BSE to macaques, although they were similar to those reported for the transmission of BASE to macaques (21). These results may therefore indicate that the L-BSE agent is more virulent in non-human primates. However, further experiments involving oral administration would be required to assess the risk of L-BSE transmission from affected cattle to humans through the consumption of beef products.

We were unable to detect PrP^{Sc} in lymphatic tissues or lymph nodes by WB or IHC. However, this does not necessarily indicate the absence of infectivity of those organs. Determination of the infectivity using inbred mice was difficult since they are reportedly insensitive or have limited sensitivity, if any, to L-BSE (25,31). The BSE/JP24 isolate could not be transmitted to three different lines of inbred mice, even 700 days after inoculation (Hagiwara, unpublished). Further analysis using transgenic mice expressing bovine or human PrP, or in vitro amplification of PrP^{Sc} by protein misfolding cyclic amplification (32,33) or quaking-induced conversion (34), would be necessary to detect trace amounts of PrP^{Sc} or infectivity in the lymphatic tissues or lymph nodes of the macaques.

The origin of atypical BSE is generally unknown, except for one H-BSE case in the United States that is considered to result from a heritable pathogenic mutation in the PrP gene (35). Epidemiological evidence (8–10,13,15) suggests that most cases of atypical BSE are found in old cattle (>8 years of age), and the relatively even birth-year distributions lead to speculation that atypical BSE is a sporadic prion disorder of cattle similar to sporadic CJD in humans (36). In this context, the conversion of BASE prion and other L-BSE prions

into C-BSE-like phenotypes during interspecies transmission to inbred mice or transgenic mice expressing ovine PrP has been reported (31,37,38). These findings are therefore of interest as regards the origin of BSE and the possible divergent evolution of prion strains.

Acknowledgments This work was supported by a grant for BSE research from the Ministry of Health, Labour and Welfare, Japan (H20-Shokuhin-Ippan-008).

Conflict of interest None to declare.

REFERENCES

1. Wells, G.A.H., Scott, A.C., Johnson, C.T., et. al. (1987): A novel progressive spongiform encephalopathy in cattle. Vet. Rec., 121, 419–420.
2. World Organization for Animal Health. Numbers of Cases of Bovine Spongiform Encephalopathy (BSE) Report in the United Kingdom. Online at 〈http://www.oie.int/eng/info/en_esbru_original.htm〉.
3. World Organization for Animal Health. Numbers of Cases of Bovine Spongiform Encephalopathy (BSE) in Farm Cattle Worldwide (excluding the United Kingdom). Online at 〈http://www.oie.int/eng/info/copie2_en_esbmonde.htm〉.
4. Will, R.G., Ironside, J.W., Zeidler, M., et al.(1996): A new variant of Creutzfeldt-Jakob disease in the UK. Lancet, 347, 921–925.
5. Collinge, J., Sidle, K.C.L., Meads, J., et al. (1996): Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature, 383, 685–690.
6. Hill, A.F., Desbruslais, M., Joiner, S., et al. (1997): The same prion strain causes vCJD and BSE. Nature, 389, 448–450.
7. Bruce, M.E., Will, R.G., Ironside, J.W., et al. (1997): Transmission to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature, 389, 498–501.
8. Casalone, C., Zanusso, G., Acutis, P., et al (2004): Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc. Natl. Acad. Sci. USA, 101, 3065–3070.
9. Biacabe, A-G., Laplanche, J-L., Ryder, S., et al. (2004): Distinct molecular phenotypes in bovine prion diseases. EMBO Rep., 5, 110–115.
10. Bushmann, A., Gretzschel, A., Biacabe, A.-G., et al. (2006): Atypical BSE in Germany-proof of transmissibility and biochemical characterization. Vet. Microbiol., 117, 103–116.
11. Baron, T.G.M., Biacabe, A-G., Bencsik, A., et al. (2006): Transmission of new bovine prion to mice. Emg. Infect. Dis., 12, 1125–1128.
12. Yamakawa, Y., Hagiwara, K., Nohtomi, K., et al. (2003): Atypical proteinase K-resistant prion protein (PrP^{res}) observed in an apparently healthy 23-month-old Holstein steer. Jpn. J. Infect. Dis., 56, 221–222.
13. Hagiwara, K., Yamakawa, Y., Sato, Y., et al. (2007): Accumulation of mono-glycosylated form-rich, plaque-forming PrP^{Sc} in the second atypical bovine spongiform encephalopathy case in Japan. Jpn. J. Infect. Dis., 60, 305–308.
14. Richt, J.A., Kunkle, R.A., Nicholson, E.M., et al. (2007): Identification of two bovine spongiform encephalopathy cases diagnosed in the United States. J. Vet. Diagn. Invest., 19, 142–154.
15. Jacobs, J.G., Langeveld, J-P.M., Biacabe, A-G., et al. (2007): Molecular discrimination of atypical bovine spongiform encephalopathy strain from geographical region spanning a wide area in Europe. J. Clin. Microbiol., 45, 1821–1829.
16. Stack, M.J., Focosi-Snyman, R., Cawthraw, S., et al. (2009): Third atypical BSE case in Great Britain with an H-type molecular profile. Vet. Rec., 14, 605–606.
17. Béringue, V., Herzog, L., Reine, F., et al. (2008): Transmission of atypical bovine prion to mice transgenic for human prion protein. Emerg. Infect. Dis., 14, 1898–1901.
18. Kong, Q., Zheng, M., Casalone, C., et al. (2008): Evaluation of the human transmission risk of an atypical bovine spongiform encephalopathy prion strain. J. Virol., 82, 3697–3701.
19. Lombardi, G., Casalone, C., D'Angelo, A., et al. (2008): Interspecies transmission of BASE induces clinical dullness and amyotrophic change. PLoS Pathog., 4, e1000075.
20. Fukuda, S., Iwamura, Y., Imamura, M., et al. (2009): Interspecies transmission of L-type-like bovine spongiform encephalopathy detected in Japan. Microbiol. Immunol., 53, 704–707.
21. Comoy, E.E., Casalone, C., Lescoutra-Etchegaray, N., et al. (2008): Atypical BSE (BASE) transmitted from asymptomatic aging cattle to a primate. PLoS ONE, 3, e3017.
22. Ministry of Health, Labour and Welfare: Results on the BSE Screening. Online at 〈http://www.mhlw.go.jp/houdou/0110/h1018-6.html〉 (in Japanese).
23. Yamanouchi, K. and Yoshikawa, Y. (2007): Bovine spongiform encephalopathy (BSE) safety measure in Japan. J. Vet. Med. Sci., 69, 1–6.
24. Yokoyama, T., Masujin, K., Yamakawa, Y., et al. (2007): Experimental transmission of two young and one suspended bovine spongiform encephalopathy (BSE) cases to bovinized transgenic mice. Jpn. J. Infect. Dis., 60, 317–320.
25. Masujin, K., Shu, Y., Yamakawa, Y., et al. (2008): Biological and biochemical characterization of L-type-like bovine spongiform encephalopathy (BSE) in Japanese black cattle. Prion, 2, 123–128.
26. Iwamaru, Y., Imamura, M., Matsuura, Y., et al. (2009): Accumulation of L-type bovine prions in peripheral nerve tissues. Emeg. Infect. Dis., 16, 1151–1154.
27. Ono, F., Terao, K., Tase, N., et al. (2011): Experimental transmission of bovine spongiform encephalopathy (BSE) to cynomolgus macaques, a non-human primate. Jpn. J. Infect. Dis., 64, 50–54.
28. Lasmézas, C.I., Deslys, J-P., Demaimay, R., et al. (1996): BSE transmission to macaques. Nature, 381, 743–744.
29. Iwata, N., Sato, Y., Higuchi, Y., et al. (2006): Distribution of PrP^{Sc} in cattle with bovine spongiform encephalopathy slaughtered at abattoirs in Japan. Jpn. J. Infect. Dis. 59, 100–107.
30. Liberski, P.P and Ironside, J.W. (2005): Neuropathology of transmissible spongiform encephalopathies (prion diseases). p. 13–48. *In* Brown, D.R. (ed.), Neurodegeneration and Prion Diseases. Springer, New York.
31. Capobianco, R., Casalone, C., Suardi, S., et al. (2007): Conversion of the BASE prion strain into the BSE strain: the origin of BSE? PLoS Pathog., 3, e31.
32. Saborio, G.P., Permanne, P. and Soto, C. (2001): Sensitive detection of prion protein by cyclic amplification of protein misfolding. Nature, 411, 810–813.
33. Chen, B., Morales, R., Barria, M.A., et al. (2010): Estimating prion concentration in fluids and tissue by quantitative PMCA. Nat. Methods, 7, 519–520.
34. Atarashi, R., Wilham, J.M., Christensen, L., et al. (2010): Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. Nat. Methods, 5, 211–212.
35. Richt, J.A. and Hall, S.M. (2008): BSE case associated with prion protein gene mutation. PLoS Pathog., 4, e1000156.
36. Brown, P., McShane, L.M., Zanusso, G., et al. (2006): On the question of sporadic or atypical bovine spongiform encephalopathy and Creutzfeldt-Jakob diseases. Emerg. Infect. Dis., 12, 1816–1821.
37. Béringue, V., Andréoletti, O., Le Dur, A., et al. (2007): A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission. J. Neurosci., 27, 6965–6971.
38. Takahashi, H., Takahashi, R.H., Hasegawa, H., et al. (1999): Characterization of antibodies raised against bovine PrP-peptides. J. Neurovirol., 5, 300–307.