ORIGINAL ARTICLE

Infectivity of Scrapie Prion Protein (PrP^{Sc}) Following *In vitro* Digestion with Bovine Gastrointestinal Microbiota

C. Scherbel[1], R. Pichner[1], M. H. Groschup[2], S. Mueller-Hellwig[3], S. Scherer[3], R. Dietrich[4], E. Maertlbauer[4] and M. Gareis[1]

[1] Institute for Microbiology and Toxicology, Federal Research Centre for Nutrition and Food, Kulmbach, Germany
[2] Institute for Novel and Emerging Diseases, Friedrich-Loeffler-Institut, Isle of Riems, Germany
[3] Unit of Microbiology, Central Research Institute for Nutrition and Food, Technical University Munich, Munich, Germany
[4] Veterinary Faculty, Institute for Hygiene and Technology of Milk, Ludwig-Maximilians University, Munich, Germany

Keywords:
Prion; gastrointestinal; degradation; rumen; infectivity; bioassays

Correspondence:
Manfred Gareis. Institute for Microbiology and Toxicology, Federal Research Centre for Nutrition and Food, E.-C. Baumann Str. 20, D-95326 Kulmbach, Germany.
Tel.: +49 9221 803 220;
Fax: +49 9221 803 331;
E-mail: manfred.gareis@bfel.de

Received for publication August 31, 2006

Summary

The influence of a complex microflora residing in the gastrointestinal tract of cattle on the prion protein plays a crucial role with respect to early pathogenesis and the potential infectivity of faeces resulting in contamination of the environment. It is unknown whether infectious prion proteins, considered to be very stable, are inactivated by microbial processes in the gastrointestinal tract of animals during digestion. In our previous study it was shown that the scrapie-associated prion protein was degraded by ruminal and colonic microbiota of cattle, as indicated by a loss of anti-prion antibody 3F4 immunoreactivity in Western blot. Subsequently, in this study hamster bioassays with the pre-treated samples were performed. Although the PrP^{Sc} signal was reduced up to immunochemically undetectable levels within 40 h of pre-treatment, significant residual prion infectivity was retained after degradation of infected hamster brain through the gastrointestinal microflora of cattle. The data presented here show that the loss of anti-prion antibody 3F4 immunoreactivity is obviously not correlated with a biological inactivation of PrP^{Sc}. These results highlight the deficiency of using Western blot in transmissible spongiform encephalopathies inactivation assessment studies and, additionally, point to the possibility of environmental contamination with faeces containing PrP^{Sc} following an oral ingestion of prions.

Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases of animals and humans (Prusiner, 1982), which are characterized by the accumulation of an abnormal disease associated and protease resistant prion protein (PrP^{Sc}), derived through conformational change from a host-encoded cellular prion protein (PrP^{C}) (Prusiner, 1999). Prions, the supposed causative agents of TSE's, are defined as 'small infectious particles which resist inactivation by procedures which modify nucleic acids' (Prusiner, 1982). They are partially, if not entirely composed of PrP^{Sc} (Prusiner, 1982), which is considered to be remarkably resistant to inactivation (Taylor, 2000). Digestion with proteinase K (PK) leads to an N-terminally truncated form of approximately 27–30 kDa detected by immunoassays in routine diagnostic (Schaller et al., 1999). The biochemical evidence for a proteolytic degradation of PrP^{Sc} by extra cellular proteases of *Bacillus* (Langeveld et al., 2003), *Streptomyces* (Hui et al., 2004) and anaerobic thermophilic prokaryotes (Tsiroulnikov et al., 2004) as well as some foodborne bacteria (Müller-Hellwig et al., 2006) has been reported. However, in these studies no information about the correlation with the levels of infectivity in bioassays is given.

An oral route of infection is commonly assumed to be important in the natural pathogenesis of bovine spongiform encephalopathy of cattle following the ingestion of

infected tissues via contaminated feed (Wilesmith et al., 1988). However, the fate of the infectious prion protein during polygastric digestion remains unclear. Feedstuffs consumed by ruminants are initially exposed to microbial fermentation in the rumen prior to gastric and intestinal digestion (Mackie and White, 1990). The polygastric digestion of ruminants in particular represents an efficient system to degrade food proteins by microbial fermentation processes in rumen and colon (Mackie and White, 1990). In a previous study, we reported on the ability of complex ruminal and colonic microbiota of cattle to decrease scrapie-associated prion protein up to immunochemically undetectable levels under physiological anaerobic conditions (Scherbel et al., 2006). Jeffrey et al. (2006) obtained similar results by incubating scrapie brain homogenate successively with alimentary tract fluids from ovine rumen, abomasum, bile and intestine *in vitro,* suggesting that the majority of scrapie prion protein of the inoculum is readily digested. Infectivity is generally related to the presence of the disease-associated prion protein (PrP^{Sc}) (McKinley et al., 1983; Caughey et al., 1997) and the study of Baier et al. (2004) has demonstrated an inactivation of 263K scrapie agent concomitant with the disappearance of PK-resistant prion protein in hamster brain homogenates. However, it is still unclear whether infectious prion proteins are inactivated by microbial processes in the gastrointestinal tract. In this study, efforts were made to determine prion infectivity of scrapie brain homogenates in hamster bioassays following digestive pre-treatment with gastrointestinal microbiota of cattle.

Materials and Methods

Experimental material

Degradation assays were performed by using normal and scrapie infected (strain 263K) hamster brain homogenates as described previously (Scherbel et al., 2006). Infected brain material was obtained from Syrian hamsters, which were inoculated intracerebrally with 20 μl of 10% scrapie-infected hamster brain homogenates in phosphate buffered saline. After 60–70 days post-infection, the animals developed clinical symptoms. The excised brains were stored at −70°C until use. A 20% homogenate of the hamster brain tissue was prepared in homogenization buffer (0.32 M sucrose, 0.5% sodium desoxycholate, 0.5% Nonidet P 40, pH 7) by using a glass douncer. All homogenates were stored in aliquots at −70°C until use. All experiments were performed in agreement with the national guidelines in a biosafety level 3** laboratory.

Rumen content and the ligatured section of the *colon ascendens* from healthy fattened beef bulls with an average age of 20 months were taken under sterile conditions immediately after slaughtering. For the control experiment the microbiota of intestinal contents was inactivated by autoclaving at 121°C for 15 min. A 10% homogenate of both the active and inactive microbiota of rumen and colon contents was prepared with sterile mineral salt buffer solution of McDougall (McDougall, 1948).

Degradation assay

Degradation assays were performed as described previously (Scherbel et al., 2006). The ruminal and colonic homogenates were filtrated in order to remove crude suspended particles, which could impair the detection of PrP^{Sc}. Samples were prepared in the ratio of 10 to 1, concerning intestinal homogenate to scrapie brain homogenate (sample AP) and brain homogenate of healthy animals (sample AN1), serving as negative control. Inactivated rumen and colon homogenates with the addition of scrapie brain homogenate represented the positive controls (sample I1). Immediately after sample preparation the references were taken and stored at −70°C until further treatment. Incubation of the samples was carried out at 37°C for up to 40 h under anaerobic and aerobic conditions.

Proteinase K treatment

Aliquots of 100 μl from each sample were digested with 10 μl Proteinase K (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 100 μg /ml for 1 h at 37°C. Reactions were stopped by addition of 20 μl 100 mM PMSF (Sigma-Aldrich), a protease inhibitor, and incubation for 15 min at room temperature (Schaller et al., 1999).

Gel electrophoresis, Western blotting and immunodetection of PrP^{Sc}

Proteins were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) together with molecular weight marker Precision Plus Protein standards, dual colour (BioRad Laboratories, Munich, Germany) and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) (Burnette, 1981; Schaller et al., 1999). Prion protein was detected by immunostaining with specific monoclonal anti-prion antibody (mab) 3F4 (Sigma-Aldrich) at 0.2 μg IgG/ml and secondary antibody conjugated to horseradish peroxidase (Dianova, Hamburg, Germany) (Schaller et al., 1999). The reaction was visualized using a highly sensitive chemiluminescence-based detection technique (ECL, Amersham Bioscience, Freiberg, Germany), following the supplier's instructions. The signals were recorded on photographic film.

Sample preparation for *in vivo* bioassay

After performing *in vitro* degradation assays, the remaining prion infectivity of the samples was analysed by hamster bioassays. To reduce the number of bacteria before inoculation, bacterial cell membranes were destroyed mechanically with small ceramic, glass and silicate beads which were expedited vigorously in the ribolyser instrument. For that purpose samples were transferred into Ribolyser Tubes Lysing Matrix E containing beads (Q-Biogene, Grünberg, Germany) and placed in a Hybaid RiboLyser^{TM} Cell Disrupter (Thermo Labsystems, Waltham, USA) for 45 s at speed 5.5. Disrupted cells were separated by short spin centrifugation (15 s, 12 000 g). The supernatant contained prion protein as detected by Western blotting (data not shown) was heated (5 min, 75°C) to inactivate microbial enzymes. With this method the number of bacteria was finally reduced from about 10^{12} to $\leq 10^{2}$ colony forming units (cfu)/ml as determined by serial dilutions plated on Standard I agar (Merck, Darmstadt, Germany).

In vivo bioassay

Each sample (30 μl) of pre-treated scrapie and normal hamster brain homogenate was inoculated intracerebrally into eight Syrian hamsters LAK/LAH (Charles River, Wilmington, MA, USA). Animals were checked every second day during the first 2-month post-inoculation. Thereafter, hamsters were assessed daily for the onset of clinical scrapie signs and clinical states were recorded as scores ranging from 0 (healthy) to 4 (severely sick). Hamsters suffering for three consecutive days from severe clinical symptoms (stage 4) were killed and the brains were taken and tested by Western blotting using mab 3F4 for the presence of PrP^{Sc} (Schaller et al., 1999). Mean incubation periods and standard errors of the mean were determined for each group and scrapie agent infectivity titres calculated on the basis of these data (Prusiner et al., 1980).

Results

Initially we demonstrated the ability of microorganisms of the gastrointestinal tract of cattle to significantly degrade PrP^{Sc} (Scherbel et al., 2006). While inactive microbiota (sample I1) did not affect the PrP^{Sc} signal in a Western blot (Fig. 1, lanes 1 and 2), following incubation for 40 h under physiological anaerobic conditions active ruminal (sample ruAPan) and colonic (sample coAPan) microbiota reduced the PrP^{Sc} signal significantly up to immunochemically undetectable levels (Fig. 1, lanes 5 and 8). Additionally, colonic microbiota (sample coAPa) eliminated all 3F4-immunoreactive material after incubation for 40 h in the presence of oxygen (Fig. 1, lane 11). As expected, the negative control with normal brain homogenate (sample AN1) showed no immunodetectable signals at all (Fig. 1, lanes 12 and 13).

In order to assess the concomitance of PK-resistant prion protein disappearance in hamster brain homogenates and the inactivation of 263K scrapie agent as shown by Baier et al. (2004), *in vivo* hamster bioassays were performed. In order to minimize the risk of bacterial brain lesions, the number of bacteria in the samples (up to 10^{12} cfu/ml) had to be reduced without impairing in parallel the PrP signal in Western blot. Common sterilization methods such as autoclaving and sterile filtration could not be used because of the negative impact on to the PrP signal in Western blot (data not shown). The reduction of the bacterial bioburden without affecting both the PrP signal and the health of the animals was achieved with

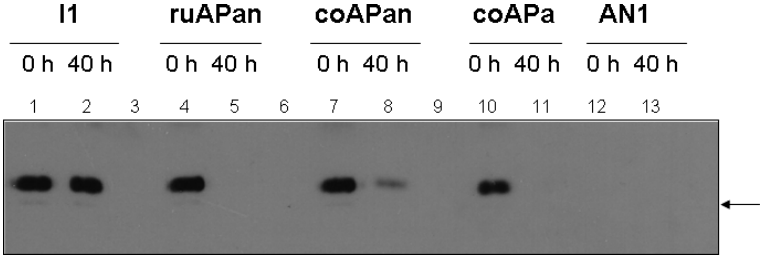

Fig. 1. *In vitro* prion protein (PrP^{Sc}) degradation studies by complex microbiota of bovine rumen and *colon ascendens* including controls. Inactivated complex intestinal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate (lanes 1 and 2) for 0 and 40 h. Complex ruminal microbiota of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 40 h (lanes 4 and 5). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under anaerobic conditions for 0 and 40 h (lanes 7 and 8). Complex microbiota from *colon ascendens* of cattle was incubated with scrapie (strain 263K) infected brain homogenate under aerobic conditions for 0 and 40 h (lanes 10 and 11). Complex intestinal microbiota of cattle was incubated with healthy hamster brain homogenate (PrP^{C}) (lanes 12 and 13) for 0 and 40 h. Arrows indicate the position of molecular-weight marker (25 kDa). Lanes 3, 6 and 9 are empty. Each lane was loaded with 25 μl of the particular PK-digested sample.

the ribolyser method, by which the bacterial counts could be decreased to levels $\leq 10^2$ cfu/ml. The results of the control demonstrated that negative effects onto the animals could be excluded (Table 1; sample AN1).

However, significant residual prion infectivity was retained after degradation of infected hamster brain through the gastrointestinal microflora of cattle. Infectivity levels in all treated or non-treated samples were determined by hamster bioassays. Measurements of incubation period length are in direct reciprocal correlation to the titre of the agent and the dilution of the inoculated sample, and can be calculated on the basis of a standard curve (Prusiner et al., 1980). Treatment of scrapie-associated prion protein under anaerobic conditions with both ruminal and colonic microflora of cattle showed no extension of mean survival time in comparison with the positive control (Table 1; samples ruAPan and coAPan). A slight increase of the mean survival time from 89.6 ± 0.72 days to 96.6 ± 4.91 was noted for the group of hamsters inoculated with samples of the colonic degradation experiment (Table 1; sample coAPa). However, this prolongation in the incubation time was due to one hamster which lacked any clinical signs and PrP^{Sc} formation most likely for artifactual reasons. Excluding this animal from the calculation diminished the one log infectivity titre reduction (corresponding in the 7 day prolongation in the medium incubation time) we conclude that the treatment had no effect on the scrapie infectivity level at all.

Discussion

Prions are characterized by an extraordinary resistance to most physical and chemical treatments classically used for inactivation of conventional pathogens. Many studies have investigated the contribution of various processes to either the reduction of infectivity or to inactivation of TSE agents (Taylor, 2000). Biological decontamination is based on enzymatic breakdown and it was shown biochemically that extra cellular proteases of *Bacillus* (Langeveld et al., 2003), *Streptomyces* (Hui et al., 2004) and anaerobic thermophilic prokaryotes (Tsiroulnikov et al., 2004) as well as some foodborne bacteria (Müller-Hellwig et al., 2006) are able to decrease PrP^{Sc} levels in infected brain homogenates of mice and hamsters. In these studies PrP^{Sc} was used as a surrogate marker for TSE infectivity. The correlation between TSE infectivity and the level of PK-resistant PrP^{Sc} served as the main biochemical evidence supporting the role of prion protein as a component of TSE agents (Diringer et al., 1983; McKinley et al., 1983). Our previous study reports on the potential of complex ruminal and colonic microbiota of cattle to decrease scrapie- associated prion protein down to levels immunochemically not detectable in Western blot (Scherbel et al., 2006). Recently, a study was published investigating the early alimentary pathogenesis of scrapie in sheep (Jeffrey et al., 2006). In accordance with our results, only trace amounts of PrP^{Sc} were detected by Western blotting after successively incubation of scrapie brain homogenates with alimentary tract fluids from ovine rumen, abomasum, bile and intestine. However, the present study shows that the loss of anti-prion antibody 3F4 immunoreactivity is obviously not correlated with a biological inactivation of PrP^{Sc}. Significant prion infectivity was present even in the absence of a Western blot signal. Similar results were also obtained in a proteolytic inactivation study of McLeod et al. (2004): The removal of all immunoreactive PrP^{Sc}, as assessed by staining with the commonly used mab 6H4, correlates poorly with the levels of infectivity measured by bioassay. Solassol et al. (2004) described the same discrepancies between Western blot and scrapie-cell assay: commercially

Table 1. Mean survival time of hamsters following intracerebral inoculation of normal and scrapie hamster brain homogenate after incubation with gastrointestinal microflora of cattle under different conditions

Sample number	Pre-treatment	Incubation conditions (40 h)	Scrapie hamster brain homogenate	Normal hamster brain homogenate	Number of affected versus inoculated hamsters	Mean survival time (days)	Standard error of the mean (days)
AN1 (negative control)	Active microflora of rumen	Anaerobic	–	+	0/8	201.0	–
I1 (positive control)	Inactive microflora of rumen	Anaerobic	+	–	8/8	89.6	0.72
ruAPan	Active microflora of rumen	Anaerobic	+	–	8/8	87.0	0.88
coAPan	Active microflora of colon	Anaerobic	+	–	8/8	88.3	1.17
coAPa	Active microflora of colon	Aerobic	+	–	7/8	96.6	4.91

available decontamination solutions showed a strong ability to reduce PrP^{Sc} levels in Western blot, but the treated tissues remained infectious as analysed in the scrapie-cell assay.

These observations raise a number of important issues for the future development of inactivation and decontamination assays for TSE agents. Conclusively, the use of Western blots or immunoassay formats may not provide an indication of the levels of prion inactivation. Therefore, the relationship between the loss of signals based on immunodetection *in vitro* and the loss of infectivity *in vivo* of PrP^{Sc} has to be comparatively analysed for each inactivation experiment. As chemical and physical processes disrupt the PK resistance but did not diminish scrapie infectivity under the same conditions (Yao et al., 2005) in accordance with our results, it challenges the conclusion that infectivity and the structure of PK resistant scrapie-associated prion protein appear to be inevitably linked (Caughey et al., 1997). This data together with previous reported results (Somerville et al., 2002) suggest that it is inappropriate to use PrP^{Sc} as surrogate marker for TSE infectivity in inactivation experiments.

Possible explanations for the observed discrepancies could be (i) that the infectivity bioassay is more sensitive than the immunoblotting, (ii) the infectivity is caused by sub-fractions of infectious PrP not detectable by immunoblotting, or (iii) the infectivity is caused by molecules or structures other than PrP.

In summary, these data highlight the deficiency of using Western blot in TSE inactivation assessment studies, and, moreover point to the fact, that the possibility of environmental contamination via infected bovine faeces can not be ruled out.

Acknowledgement

This work was supported mainly by the Bavarian Research Cooperation FORPRION (Grant No. 1205TG81LMU19a) and also by the German Ministries of Science, Research and Art as well as of Health, Food and Consumer Protection. Further support was given by the European Commission (NoE *Neuroprion*). We gratefully thank Mrs Bärbel Hammerschmitt and Mrs Liane Weber for excellent technical assistance, Bernhard Schurr and the staff of the abattoir in Kulmbach for collaboration.

References

Baier, M., A. Schwarz, and M. Milke, 2004: Activity of alkaline cleaner in the inactivation of scrapie agent. *J. Hosp. Infect.* 57, 80–84.

Burnette, W. N., 1981: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112, 195–203.

Caughey, B., G. J. Raymond, D. A. Kocisko, and P. T. Landsbury Jr , 1997: Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies. *J. Virol.* 71, 4107–4110.

Diringer, H., H. Gelderblom, H. Hilmert, M. Ozel, C. Edelbluth, and R. H. Kimberlin, 1983: Scrapie infectivity, fibrils and low molecular weight protein. *Nature* 306, 476–478.

Hui, Z., H. Doi, H. Kanouchi, Y. Matsuura, S. Mohri, Y. Nonomura, and T. Oka, 2004: Alkaline serine protease produced by *Streptomyces* sp. degrades PrP(Sc). *Biochem. Biophys. Res. Commun.* 321, 45–50.

Jeffrey, M., L. Gonzalez, A. Espenes, C. M. Press, S. Martin, M. Chaplin, L. Davis, T. Landsverk, C. MacAldowie, S. Eaton, and G. McGovern, 2006: Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep. *J. Pathol.* 209, 4–14.

Langeveld, J. P., J. J. Wang, D. F. Van de Wiel, G. C. Shih, G. J. Garssen, A. Bossers, and J. C. Shih, 2003: Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J. Infect. Dis.* 188, 1782–1789.

Mackie, R. I., and B. A. White, 1990: Symposium: rumen microbial ecology and nutrition. *J. Diary. Sci.* 73, 2971–2995.

McDougall, E. I., 1948: Studies on ruminant salvia. I. The composition and output of sheep's salvia. *J. Biochem.* 43, 99–109.

McKinley, M. P., D. C. Bolton, and S. B. Prusiner, 1983: A protease-resistant protein is a structural component of the scrapie prion. *Cell* 35, 57–62.

McLeod, A. H., H. Murdoch, J. Dickinson, M. J. Dennis, G. A. Hall, C. M. Buswell, J. Carr, D. M. Taylor, J. M. Sutton, and N. D. Raven, 2004: Proteolytic inactivation of the bovine spongiform encephalopathy agent. *Biochem. Biophys. Res. Commun.* 317, 1165–1170.

Müller-Hellwig, S., M. H. Groschup, R. Pichner, M. Gareis, E. Märtlbauer, S. Scherer, and M. J. Loessner, 2006: Biochemical evidence for the proteolytic degradation of infectious prion protein PrPSc in hamster brain homogenates by food-borne bacteria. *Syst. Appl. Microbiol.* 29, 165–171.

Prusiner, S. B., 1982: Novel proteinaceous infectious particles cause scrapie. *Science* 216, 136–144.

Prusiner, S. B., 1999: An introduction to prion biology and diseases. In: Prusiner, S. B. (ed), Prion Biology and Diseases, pp. 1–66. Cold Spring Harbor Laboratory Press, New York.

Prusiner, S. B., D. F. Groth, S. P. Cochran, F. R. Masiarz, M. P. McKinley, and H. M. Martinez, 1980: Molecular properties, partial purification, and assay by incubation period measurements of the hamster scrapie agent. *Biochem.* 19, 4883–4891.

Schaller, O., R. Fatzer, and M. Stack, 1999: Validation of a Western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). *Act. Neuropathol.* 227, 680–685.

Scherbel, C., R. Pichner, M. H. Groschup, S. Müller-Hellwig, S. Scherer, R. Dietrich, E. Märtlbauer, and M. Gareis, 2006: Degradation of scrapie associated prion protein (PrP^{Sc}) by the gastrointestinal microbiota of cattle. *Vet. Res.* 37, 695–703.

Solassol, J, M. Arlotto, and S. Lehmann, 2004: Detection of prion after decontamination procedures: comparative study of standard Western blot, filter retention and scrapie-cell assay. *J. Hosp. Infect.* 52, 156–161.

Somerville, R. A., R. C. Oberthur, U. Havekost, F. MacDonald, D. M. Taylor, and A. G. Dickinson, 2002: Characterisation of thermodynamic diversity between transmissible spongiform encephalopathy agent strains and its theoretical implications. *J. Biol. Chem.* 277, 11084–11089.

Taylor, D. M., 2000: Inactivation of transmissible degenerative encephalopathy agents: a review. *Vet. J.* 159, 10–17.

Tsiroulnikov, K., H. Rezai, E. Bonch-Osmolovskaya, P. Nedkov, A. Gousterova, V. Cueff, A. Godfroy, G. Barbier, F. Metro, J. M. Chobert, P. Clayette, D. Dormont, J. Grosclaude, and T. Haertle, 2004: Hydrolysis of the amyloid prion protein and nonpathogenic
meat and bone meal by anaerobic thermophilic prokaryotes and *Streptomyces* subspecies. *J. Agric. Food. Chem.* 52, 6353–6360.

Wilesmith, J. W., G. A. Wells, M. P. Cranwell, and J. B. Ryan, 1988: Bovine spongiform encephalopathy: epidemiological studies. *Vet. Rec.* 123, 638–644.

Yao, H. L., J. Han, J. M. Gao, J. Zhang, B. Y. Zhang, Y. J. Guo, K. Nie, C. Gao, X. F. Wang, and X. P. Dong, 2005: Comparative study of the effects of several chemical and physical treatments on the activity of protease resistance and infectivity of scrapie strain 263K. *J. Vet. Med. B* 52, 437–443.