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PrP^d accumulation in organs of ARQ/ARQ sheep experimentally infected with BSE by peripheral routes

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To study the pathogenesis of bovine spongiform encephalopathy infection in small ruminants, two Lacaune sheep with the AA₁₃₆RR₁₅₄QQ₁₇₁ and one with the AA₁₃₆RR₁₅₄RR₁₇₁ genotype for the prion protein, were inoculated with a brain homogenate from a French cattle BSE case by peripheral routes. Sheep with the ARQ/ARQ genotype are considered as susceptible to prion diseases contrary to those with the ARR/ARR genotype. The accumulation of disease-associated prion protein (PrP^d) was analysed by biochemical and immunohistochemical methods. No PrP^d accumulation was detected in samples from the ARR/ARR sheep 2 years post inoculation. In the two ARQ/ARQ sheep that had scrapie-like clinical symptoms, PrP^d was found in the central, sympathetic and enteric nervous systems and in lymphoid organs. Remarkably, PrP^d was also detected in some muscle types as well as in all peripheral nerves that had not been reported previously thus revealing a widespread distribution of BSE-associated PrP^d in sheep tissues.

Keywords: ovine BSE, prion, scrapie, sheep

Running title: widespread distribution of PrP^d in sheep infected with BSE.

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INTRODUCTION

The bovine spongiform encephalopathy (BSE) agent, linked to the variant Creutzfeldt-Jakob disease in humans (Bruce *et al.*, 1997), has been experimentally transmitted to sheep (Foster

et al., 1993), without clinical distinction from natural scrapie. The possible presence of this agent within sheep flocks used for human consumption is therefore of considerable concern. It has recently been demonstrated in France that a major risk factor for introduction of transmissible spongiform encephalopathies (TSE) in a flock is linked to the use of proprietary concentrates and milk replacers (Philippe *et al.*, 2005), which have been implicated in the BSE epidemic in cattle (Wilesmith *et al.*, 1992). Furthermore, the BSE agent was recently identified in a naturally infected French goat (Eloit *et al.*, 2005) and this reinforces the possible presence of the BSE agent in the sheep and goat flocks.

In sheep, the development of prion disease is complex and depends on several factors such as the genotype and breed of animals as well as the nature of the infectious agent. Thus, polymorphism of the prion protein PrP gene that predominantly determines scrapie or BSE susceptibility are linked to variations of codons 136, 154 and 171. The V₁₃₆R₁₅₄Q₁₇₁/V₁₃₆R₁₅₄Q₁₇₁ genotypes are associated with a very high susceptibility to scrapie (Hunter 1997) whereas the ARR/ARR animals are more resistant to scrapie (Hunter 2003) even though cases of scrapie have been reported in this genotype (Buschmann *et al.*, 2004); (Ikeda *et al.*, 1995). The ARQ/ARQ genotype, largely represented in flocks, and associated with scrapie susceptibility, would lead to an increased susceptibility to BSE (Houston *et al.*, 2003a).

To study the pathogenesis of BSE in small ruminants, Lacaune sheep were inoculated with the BSE agent. The accumulation of the disease-associated prion protein, PrP^d, the most specific marker of the disease (Bolton *et al.*, 1982), was investigated using three complementary methods that allowed us to obtain both qualitative and quantitative results (Madec *et al.*, 2004) allowing the identification of the tissues that may represent a risk for consumption.

Material and Methods

Animals

In our study, two ARQ/ARQ Lacaune sheep were inoculated either by intra-peritoneal (SB1 sheep) or intra-splenic (SB3 sheep) routes with brain homogenate from a French BSE-affected cow. One sheep naturally died at 672 days post inoculation (dpi) and the other was euthanized 1444 dpi. Both sheep had clinical signs of neurological disorders and had molecular characteristics of PrP^d consistent with BSE infection (Houston *et al.*, 2003a); (Lezmi *et al.*, 2004). The other Lacaune sheep with the ARR/ARR genotype (SB2) was inoculated by the intra-peritoneal route and was sacrificed at 673 dpi.

Samples from healthy sheep 2 years of age having the ARR/ARR and ARR/ARQ genotypes were used as negative controls. The negative status of these animals was confirmed by checking the absence of PrP^d in the central nervous system (CNS) and tonsils.

Immunohistochemistry (IHC)

IHC allowing the identification of PrP^d at the cellular level with high sensitivity was performed as described in previous studies (Lezmi *et al.*, 2003); PrP^d deposits appeared *in situ* as brown or black deposits using DAB alone or intensified with NiCl₂.

Western-blot (WB)

Following a WB procedure previously described (Biacabe *et al.*, 2004), PrP^d detection was performed using anti-PrP mAbs Bar233 and peroxidase-conjugated anti-mouse IgG (Southern Biotechnology Associates). This method has the advantage of identifying with high specificity the proteinase K (PK) resistant form of PrP^d. However, the WB analysis is less sensitive than the ELISA method, and as all samples were not in sufficient quantities for both biochemical analysis, samples were tested by ELISA in priority, as this method allows quantification of PrP^d.

ELISA

ELISA was performed with the extraction kit 'Platelia BSE Bio-Rad' currently used for TSE diagnosis (Grassi *et al.*, 2001). For each plate an internal standard was used, i.e. ovine recombinant prion (^{rec}PrP) purified as previously described (Betemps *et al.*, 2001). ELISA detection was performed using SAF34 anti-PrP mAbs as capture antibodies and acetylcholinesterase-conjugated Bar224 anti-PrP mAbs as a tracer.

Results and Discussion

For all samples analysed from the ARR/ARR sheep, no PrP^d was detected by any of the three PrP^d detection methods used (table 1). This result correlates with the higher genetic resistance to TSE associated with this genotype naturally affected with scrapie (Elsen *et al.*, 1999) or orally infected with the BSE agent (Jeffrey *et al.*, 2001). However, resistance of the ARR/ARR sheep challenged with TSE infection is not considered complete since natural scrapie cases have been reported in sheep with this genotype (Buschmann *et al.*, 2004); (Ikeda *et al.*, 1995) (French surveillance program; unpublished data). Furthermore, BSE has been transmitted to ARR/ARR sheep by the intra-cerebral route (Houston *et al.*, 2003b).

In both ARQ/ARQ sheep, the CNS (including retina), the lymphoid system and the autonomous nervous system were identified by each method as major sites of PrP^d accumulation (table 1 ; figure 1) and were also described earlier by other groups in experimentally BSE affected sheep (Foster *et al.*, 2001; Jeffrey *et al.*, 2001) as well as in naturally scrapie-affected sheep (Jeffrey *et al.*, 2001; van Keulen *et al.*, 1999). In the CNS, the quantities of abnormal PrP, expressed as equivalent in ^{rec}PrP, were estimated by ELISA at up to 13000 ng/g of brainstem tissue. Comparatively, the levels found in 13 ARQ/ARQ or ARQ/VRQ sheep clinically affected with natural scrapie averaged 40 000 ± 20 000 ng of PrP^d/g of CNS tissues. Lymphoid organs accumulated lower levels of PrP^d and large quantities of material were required to detect a signal by WB in the mandibular or iliac medial

lymph nodes (LN) of SB3 (Figure 1). In the spleen of SB1 and SB3, 46 and 2 ng equivalent of PrP^d/g of tissue were detected, respectively. In the ileum, 232 ng equivalent of PrP^d /g of tissue was detected and correlated with a higher number and size of germinal centres when compared to spleen or iliac LN. The mean quantity of PrP^d in the CNS was 187 and 36 fold greater than the quantities determined respectively in spleen and in the intestine.

Qualitatively, different types of PrP^d deposits in the brain were identified from the frontal cortex to the lumbar spinal cord. These PrP^d deposits were mainly identical to those previously identified in scrapie- or BSE-affected sheep (Gonzalez *et al.*, 2002; Ryder *et al.*, 2001). In the retina, PrP^d accumulation was mainly detected in the ganglionar layer (1), intern (2) and extern (4) plexiform layers (numbers corresponding to the different layers in the retina, figure 2a). Interestingly, in the enteric nervous system of ARQ/ARQ sheep, PrP^d was detected associated with neurons (figure 2g) as well as in the coeliac ganglia in which intra- and peri-neuronal PrP^d deposits were visualized (figure 2h). In the adrenal gland, two types of PrP^d accumulation were observed as dense intracellular or synaptic-like deposits (figure 2i).

In lymphoid organs, PrP^d was detected in germinal centres of secondary lymphoid follicles, in follicular dendritic cells and in tingible body macrophages (figure 2j). PrP^d was also detected in cells with a morphology consistent with macrophages in the subcapsular sinus of some lymph nodes (figure 2j, arrowhead, table 1*). These observations are in agreement with previous results obtained both in sheep naturally affected with scrapie (Ersdal *et al.*, 2005); (Jeffrey *et al.*, 2000); (Lezmi *et al.*, 2001) and in experimentally BSE-infected sheep (Lezmi *et al.*, 2001); (Jeffrey *et al.*, 2001). Interestingly, not all germinal centres were labelled for PrP^d; this partial absence of labelling in germinal centres (as in tonsils) was not observed in samples from 13 natural scrapie-infected sheep in which all lymphoid germinal centres were positively labelled for PrP^d. This agreed with data describing an early and systematic immune system involvement in lambs affected with scrapie (Andreoletti *et al.*, 2000) which was not a

feature of BSE agent infection in sheep during the first passage (Jeffrey *et al.*, 2001; Martin *et al.*, 2005).

In our study, as opposed to previous published results, in both ARQ/ARQ sheep, PrP^d was detected by IHC in all motor nerves and associated with Schwann's cells (figure 2d, e). PrP^d deposits were similarly detected in all other tissue samples containing peripheral nerves, most notably in nerves in muscle samples. This observation was not reported in other studies with sheep BSE (Foster *et al.*, 2001; Jeffrey *et al.*, 2001). However, we observed the same type of deposits in two other sheep (ARQ/VRQ) naturally affected with scrapie (data not shown) and two previous articles report similar data in sheep with natural scrapie (Archer *et al.*, 2004); (Groschup *et al.*, 1999).

PrP^d presence was also identified in striated muscles for both ARQ/ARQ sheep. These deposits were associated with neuromuscular spindles that are highly innervated structures made of groups of myocytes surrounded by a thin fibrous capsule (figure 2k, l) and are a specialized subset of myocytes implicated in proprioception. In the tongue of sheep, the accumulation of PrP^d in these structures was less evident. Only one study reported the PrP^d presence in the muscle of sheep affected with scrapie using IHC and ELISA (Andreoletti *et al.*, 2004). Here, sampling and analysis of different muscles were not systematic and thus the ELISA/IHC results were not correlated. However, the accumulation in muscle tissue of PrP^d in sheep affected with scrapie is not systematic (Andreoletti *et al.*, 2004). Recently, pathological prion protein was detected in muscles of hamsters and mice infected with rodent-adapted BSE or vCJD (Thomzig *et al.*, 2006). Previously, other studies failed to detect prion in nerves and muscles of BSE- or scrapie-infected sheep (Foster *et al.*, 2001); (Hamir *et al.*, 2004) possibly relying on the use of different pre-treatments and antibodies.

In conclusion, we have shown that the inoculation of the BSE agent of French origin by peripheral routes to Lacaune sheep lead to the development of the clinical disease only in

ARQ/ARQ sheep. The distribution of PrP^d in ARQ/ARQ sheep infected with BSE was very similar to that described in natural scrapie. Overall, we demonstrated for the first time the presence of PrP^d in muscles and nerves of sheep infected experimentally with BSE agent, which stresses the potential risk for humans related to consumption of sheep products from sheep naturally infected with BSE.

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REFERENCES

- Andreoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, vanKeulen L, Schelcher F, Elsen J M, Lantier F (2000) Early accumulation of PrP^{Sc} in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. *J Gen Virol* **81**: 3115–3126.
- Andreoletti O, Simon S, Lacroux C, Morel N, Tabouret G, Chabert A, Lugan S, Corbiere F, Ferre P, Foucras G, Laude H, Eychenne F, Grassi J, Schelcher F (2004) PrP^{Sc} accumulation in myocytes from sheep incubating natural scrapie. *Nat Med* **10**: 591–593.
- Archer F, Bachelin C, Andreoletti O, Besnard N, Perrot G, Langevin C, Le Dur A, Vilette D, Van Evercooren A, Vilotte J, Laude H (2004) Cultured peripheral neuroglial cells are highly permissive to sheep prion infection. *J Virol* **78**: 482–490.

- Betemps D, Baron T (2001) Molecular specificities of antibodies against ovine and murine recombinant prion proteins. *Biochem Biophys Res Com* **281**: 101–108.
- Biacabe A G, Laplanche J L, Ryder S, Baron T (2004) Distinct molecular phenotypes in bovine prion diseases. *EMBO Reports* **5**: 110–115.
- Bolton D C, McKinley M P, Prusiner S B (1982) Identification of a Protein That Purifies with the Scrapie Prion. *Science* **218**: 1309–1311.
- Bruce M E, Will R G, Ironside J W, McConnell I, Drummond D, Suttie A, McCordle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock C J (1997) Transmission to mice indicate that "new variant" CJD is caused by the BSE agent. *Nature* **389**: 498–501.
- Buschmann A, Luhken G, Schultz J, Erhardt G, Groschup M (2004) Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR). *J Gen Virol* **85**: 2727–2733.
- Eloit M, Adjou K, Couplier M, Fontaine J, Hamel R, Lilin T, Messiaen S, Andreoletti O, Baron T, Bencsik A, Biacabe A, Beringue V, Laude H, Le Dur A, Vilotte J, Comoy E, Deslys J, Grassi J, Simon S, Lantier F, Sarradin P (2005) BSE agent signatures in a goat. *Vet Rec* **156**: 523–524.
- Elsen J-M, Amigues Y, Schelcher F, Ducrocq V, Andréoletti O, Eychenne F, Vu Tien Khang J, Poivey J-P, Lantier F, Laplanche J-L (1999) Genetic susceptibility and transmission factors in scrapie: Detailed analysis of an epidemic in a closed flock of Romanov. *Arch Virol* **144**: 431–445.
- Ersdal C, Ulvund M, Espenes A, Benestad S, Sarradin P, Landsverk T (2005) Mapping PrPSc propagation in experimental and natural scrapie in sheep with different PrP genotypes. *Vet Pathol* **42**: 258–274.

- Foster J D, Hope J, Fraser H (1993) Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet Record* **133**: 339–341.
- Foster J D, Parnham D W, Hunter N, Bruce M (2001) Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. *J Gen Virol* **82**: 2319–2326.
- Gonzalez L, Martin S, BegaraMcGorum I, Hunter N, Houston F, Simmons M, Jeffrey M (2002) Effects of agent strain and host genotype on PrP accumulation in the brain of sheep naturally and experimentally affected with scrapie. *J Comp Pathol* **126**: 17–29.
- Grassi J, Comoy E, Simon S, Créminon C, Frobert Y, Trapman S, Schimmel H, Hawkins S A C, Moynagh J, Deslys J P, Wells G A H (2001) Rapid test for the preclinical postmortem diagnosis of BSE in central nervous system tissue. *Vet Record* **149**: 577–582.
- Groschup M H, Beekes M, McBride P A, Hardt M, Hainfellner J A, Budka H (1999) Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie. *Acta Neuropathol* **98**: 453–457.
- Hamir A, Miller J, Cutlip R (2004) Failure to detect prion protein (PrPres) by immunohistochemistry in striated muscle tissues of animals experimentally inoculated with agents of transmissible spongiform encephalopathy. *Vet Pathol* **41**: 78–81.
- Houston E F, Gravenor M B (2003a) Clinical signs in sheep experimentally infected with Scrapie and BSE. *Vet. Record* **152**: 333–334.
- Houston F, Goldmann W, Chong A, Jeffrey M, González L, Foster J, Parnham D, Hunter N (2003b) BSE in sheep bred for resistance to infection. *Nature* **423**: 98.
- Hunter N (1997) PrP genetics in sheep and the application for scrapie and BSE. *Trends Microbiol.* **5**: 331–334.

- Hunter N (2003) Scrapie and experimental BSE in sheep. *British Medical Bulletin* **66**: 171–183.
- Ikeda T, Horiuchi M, Ishiguro N, Muramatsu Y, Uwe G G, Shinagawa M (1995) Amino acid polymorphisms of PrP with references to onset of scrapie in Suffolk and Corriedale sheep in Japan. *J Gen Virol* **76**: 2577–2581.
- Jeffrey M, McGovern G, Martin S, Goodsir C M, Brown K L (2000) Cellular and sub-cellular localisation of PrP in the lymphoreticular system of mice and sheep. *Arch Virol* **23**–38.
- Jeffrey M, Ryder S, Martin S, Hawkins S A C, Terry L, BerthelinBaker C, Bellworthy S J (2001) Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera. *J Comp Pathol* **124**: 280–289.
- Lezmi S, Bencsik A, Baron T (2001) CNA42 monoclonal antibody identifies FDC as PrPsc accumulating cells in the spleen of scrapie affected sheep. *Vet Immunol Immunopathol* **82**: 1–8.
- Lezmi S, Bencsik A, Monks E, Petit T, Baron T (2003) First case of feline spongiform encephalopathy in a captive cheetah born in France: PrPsc analysis in various tissues revealed unexpected targeting of kidney and adrenal gland. *Histochem Cell Biol* **119**: 415–422.
- Lezmi S, Martin S, Simon S, Comoy E, Bencsik A, Deslys J P, Grassi J, Jeffrey M, Baron T (2004) Comparative molecular analysis of the abnormal prion protein in field scrapie cases and experimental bovine spongiform encephalopathy in sheep by use of Western blotting and immunohistochemical methods. *J Virol* **78**: 3654–3662.
- Madec J, Simon S, Lezmi S, Bencsik A, Grassi J, Baron T (2004) Abnormal prion protein in genetically resistant sheep from a scrapie-infected flock. *J Gen Virol* **85**.

- Philippe S, Ducrot C, Roy P, Remontet L, Jarrige N, Calavas D (2005) Sheep feed and scrapie, France. *Emerg Infect Dis* **11**: 1274–129.
- Ryder S J, Spencer Y I, Bellerby P J, March S A (2001) Immunohistochemical detection of PrP in the medulla oblongata of sheep: the spectrum of staining in normal and scrapie-affected sheep. *Vet Record* **148**: 7–13.
- Thomzig A, Cardone F, Kruger D, Pocchiari M, Brown P, Beekes M (2006) Pathological prion protein in muscles of hamsters and mice infected with rodent-adapted BSE or vCJD. *J Gen Virol* **87**: 251–254.
- van Keulen L J M, Schreuder B E C, Vromans M E W, Langeveld J P M, Smits M A (1999) Scrapie-associated Prion Protein in the Gastro-intestinal Tract of Sheep with Natural Scrapie. *J Comp Pathol* **121**: 55–63.
- Wilesmith J W, Ryan J B M, Hueston W D (1992) Bovine spongiform encephalopathy: case-control studies of calf feeding practices and meat and bonemeal inclusion in proprietary concentrates. *Res Vet Sc* **52**: 325–331.

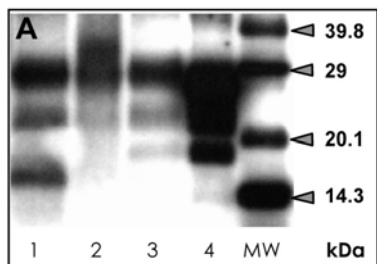


Figure 1. Detection of PrP^d by Western blot-ARQ/ARQ sheep. Representative pattern of PrP^d observed in different tissues. Lanes 1 and 2: mandibular and iliac-medial lymph nodes, respectively, lane 3: adrenal gland, lane 4: cortex. All homogenates were prepared from sheep SB3.

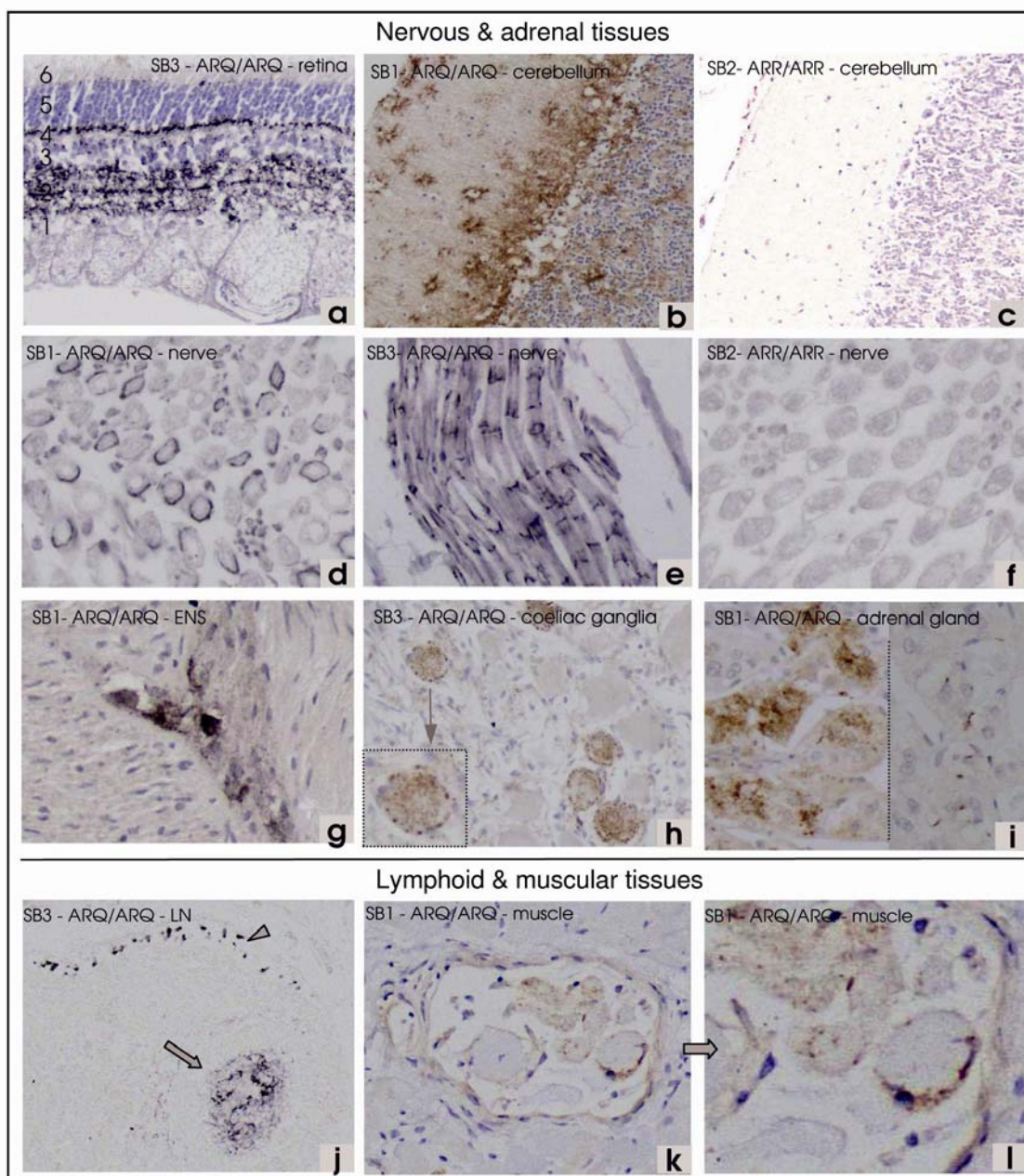


Figure 2. immunodetection of PrP^d in various tissues. PrP^d accumulation was detected in the retina (**a**, black deposits, x200) and in the cerebellum of both ARQ/ARQ sheep (**b**, brown deposits) but not in the ARR/ARR sheep (**c**). PrP^d was also detected in the ARQ/ARQ sheep in sciatic nerve (**d–e**, black deposits, x400, x200) but neither in the ARR/ARR sheep nor in healthy controls (**f**, x200). In the enteric nervous system of ARQ/ARQ sheep, PrP^d was detected associated to neurons (**g**, black deposits, x200) as well as in the coeliac ganglia in

which intra and peri-neuronal PrP^d deposits were visualized (**h**, brown deposits, x200; higher magnification in the inset). In the adrenal gland, two types of PrP^d accumulation were observed, dense intracellular and synaptic-like (**i**, brown deposits, x400). In lymph nodes, the PrP^d accumulation was mainly detected in germinal centers (arrow) (**j**, black deposits, x100). In the muscle PrP^d accumulation was observed associated to neuro-muscular spindle (**k-l**, brown deposits, x100 and x400).

Table 1. Detection of PrP^d by immunohistochemistry (IHC), ELISA and Western blot (WB). For ELISA measurements, samples were stated positive or negative by reference to a cut-off value calculated as the mean of the measurements made on the negative controls plus 3-fold the value of the standard deviation calculated on negative controls. A “grey area” was defined between the value of the cut off and the mean of the negative controls plus 2-fold the value of the standard variation (Table 1, ‘+/-’ results). PrP^d accumulation was also quantified (values in brackets, ng/g of tissue). For each plate, at least three negative controls chosen in function of the studied tissue were deposited in duplicate.

The intensity of PrP^d immunolabelling was estimated for IHC and Western blot according to the scoring: - no labelling; +/- trace of light labelling; + light labelling; ++ moderate labelling; +++ intense labelling; and ++++ very strong labelling. Basing on the results obtained with the different methods, a final conclusion (con.) was proposed for each tissue on the presence of PrP^d.

CNS: central nervous system, ENS: enteric nervous system, PP: Peyer’s patches, gg: ganglia, sp. cord: spinal cord, nt: not tested, na: not available, +*: PrP^d accumulation in under-capsular area in some lymph nodes (LN).

TABLE 1

| Samples | SB1 ARQ/ARQ | | | | SB3 ARQ/ARQ | | | | SB2 ARR/ARR | | | |
|-------------------------|-------------|-----------|------|------|-------------|-----------|------|------|-------------|-------|----|------|
| | IHC | ELISA | WB | Con. | IHC | ELISA | WB | Con. | IHC | ELISA | WB | Con. |
| CNS | | | | | | | | | | | | |
| Frontal cortex | +++ | + (4526) | ++++ | ++++ | +++ | + | ++++ | ++++ | - | - | nt | - |
| Cerebellum | +++ | + | ++++ | ++++ | +++ | + (5372) | ++++ | ++++ | - | - | nt | - |
| Brain stem | ++++ | + | ++++ | ++++ | ++++ | + (13266) | ++++ | ++++ | - | - | nt | - |
| Cervical sp. cord | +++ | + (2959) | ++++ | ++++ | +++ | + (2081) | ++++ | ++++ | - | - | nt | - |
| Thoracic sp. cord | +++ | + (1172) | ++++ | ++++ | +++ | + (2439) | ++++ | ++++ | - | - | nt | - |
| Lumbar sp. cord | +++ | + (4819) | ++++ | ++++ | +++ | + (4002) | ++++ | ++++ | - | - | nt | - |
| Lymphoid organs | | | | | | | | | | | | |
| Tonsils | ++ | na | ++ | ++ | ++ | + (107) | na | ++ | - | na | - | - |
| Soft palate | na | na | na | - | ++ | na | na | ++ | na | na | na | - |
| Retropharyngeal LN | ++ | na | na | ++ | + | - | nt | + | - | na | na | - |
| Mandibular LN | + | +/- | - | + | + | + (2 ± 1) | + | + | - | - | nt | - |
| Thoracic LN | + | na | na | + | na | na | na | - | na | na | na | - |
| Mediastinal LN | + | - | nt | + | + | - | nt | + | - | - | nt | - |
| Ruminal LN | na | na | na | - | + | na | na | + | na | na | na | - |
| Ileal LN | + | na | na | + | - | + (5) | nt | + | - | na | na | - |
| Caecal LN | + | na | na | + | + | - | - | + | - | - | nt | - |
| Spiral colon LN | + | + (1 ± 0) | nt | + | - | +/- | nt | +/- | na | - | nt | - |
| Ilio-femoral LN | + | +/- | nt | + | + | na | na | + | - | - | nt | - |
| Iliac-medial LN | - | - | nt | - | + | + (12) | +/- | + | - | - | nt | - |
| Spleen | +/+ | + (46) | + | ++ | + | + (2) | - | + | - | - | nt | - |
| Prescapular LN | + | + (12) | + | + | + | na | na | + | - | - | nt | - |
| Precurral LN | na | na | na | - | + | + (5) | +/- | + | - | na | na | - |
| Popliteal LN | + | + (2 ± 1) | - | + | + | + (25) | +/- | + | - | - | nt | - |
| Digestive tract | | | | | | | | | | | | |
| Oesophagus ENS | na | na | na | - | - | - | nt | - | na | na | na | - |
| Rumen ENS | na | na | na | - | - | - | nt | - | na | na | na | - |
| Reticulum ENS | na | na | na | - | - | - | nt | - | na | na | na | - |
| Omasum ENS | na | na | na | - | - | - | nt | - | na | na | na | - |
| Abomasum ENS | na | na | na | - | + | - | nt | + | na | na | na | - |
| Duodenum ENS | + | - | - | + | - | - | nt | - | - | - | nt | - |
| Jejunum ENS | na | na | na | - | - | - | nt | + | - | na | na | - |
| Ileum ENS | + | + | + | + | + | - | nt | + | nt | nt | nt | - |
| Ileum PP | ++ | + (232) | + | ++ | + | - | nt | + | - | na | na | - |
| Ileo-caecum ENS | + | na | na | + | + | na | na | + | - | na | na | - |
| Ileo-caecum PP | ++ | na | na | ++ | ++ | - | nt | ++ | - | - | na | - |
| Caecum ENS | + | +/- | +/- | + | - | - | nt | - | nt | nt | nt | - |
| Spiral colon ENS | + | + | + | + | - | - | nt | - | - | nt | nt | - |
| Spiral colon PP | + | + (12) | +/- | + | na | - | nt | - | - | - | nt | - |
| Rectum ENS | - | na | na | - | - | - | nt | - | na | na | na | - |
| Per. Nerv. Syst. | | | | | | | | | | | | |
| Cervical.Cra.gg | ++ | na | na | ++ | + | na | na | + | - | na | na | - |
| Coeliac ganglia | ++ | na | na | ++ | + | na | na | + | - | na | na | - |
| Sympathic gg | na | na | na | - | +/- | na | na | +/- | - | na | na | - |
| Vagus nerve | +/- | - | - | +/- | +/- | - | nt | +/- | - | na | na | - |
| Splanchnic nerve | +/- | na | na | +/- | +/- | na | na | +/- | - | na | na | - |
| Sciatic nerve | + | - | - | + | + | - | nt | + | - | - | nt | - |
| Brachial plexus | + | - | nt | + | + | - | nt | + | - | - | nt | - |
| Lingual nerve | na | na | na | - | + | + (2) | nt | + | na | na | na | - |
| Phrenic nerve | na | na | na | - | na | na | na | - | - | na | na | - |

TABLE 1 (next)

| Samples | SB1 - ARQ/ARQ | | | | SB3 - ARQ/ARQ | | | | SB2 - ARR/ARR | | | |
|----------------------|---------------|---------|----|------|---------------|--------|----|------|---------------|-------|----|------|
| | IHC | ELISA | WB | Con. | IHC | ELISA | WB | Con. | IHC | ELISA | WB | Con. |
| Eyes | | | | | | | | | | | | |
| Aqueous humour | na | + (35) | nt | + | na | na | na | | na | - | nt | - |
| Cornea | - | - | nt | - | - | +/- | nt | +/- | - | na | na | - |
| Lens | - | - | - | - | - | - | - | - | - | na | na | - |
| Iris | - | - | nt | - | - | +/- | - | +/- | - | na | na | - |
| Optic nerve | - | - | nt | - | - | - | nt | - | - | na | na | - |
| Retina | +++ | + (7) | nt | +++ | +++ | + (15) | - | +++ | - | na | na | - |
| Vitreous humour | na | + (29) | - | + | na | - | - | - | na | - | nt | - |
| | | | | | | | | | | | | |
| Other samples | | | | | | | | | | | | |
| Adrenal gland | ++ | + (111) | + | ++ | + | + (53) | + | + | - | - | nt | - |
| Urinary bladder | na | na | na | | - | - | nt | - | na | na | na | |
| Carotid | na | na | na | | - | - | nt | - | na | na | na | |
| Gallbladder | na | na | na | | - | na | na | - | na | na | na | |
| Heart, auricle | - | na | na | - | - | na | na | - | na | na | na | |
| Heart, ventricle | - | - | - | - | - | - | nt | - | - | na | na | - |
| Kidney | - | - | nt | - | - | - | nt | - | - | - | nt | - |
| Liver | - | - | - | - | - | - | nt | - | - | - | nt | - |
| Lung | - | na | na | - | - | - | nt | - | - | na | na | - |
| Mouth mucosa | na | na | na | | - | na | na | - | na | na | na | |
| Ovary | na | na | na | | - | - | nt | - | - | na | na | - |
| Pancreas | na | na | na | | - | - | nt | - | na | na | na | |
| Salivary gland | - | na | na | - | - | - | nt | - | - | na | na | - |
| Skin | - | na | na | - | - | - | nt | - | na | na | na | |
| Skin lesion | - | na | na | - | na | na | na | | na | na | na | |
| Striated muscles | + | - | nt | + | + | - | nt | + | nt | nt | nt | |
| Thyroid | na | na | na | | - | - | nt | - | na | na | na | |
| Tongue | + | na | na | + | + | - | nt | + | na | na | na | |
| Trachea | na | na | na | | - | + (21) | nt | + | na | na | na | |
| Uterus | na | na | na | | - | - | nt | - | - | na | na | - |