Detection of classical and atypical/Nor98 scrapie by the paraffin-embedded tissue blot method

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Summary

The paraffin-embedded tissue (PET) blot method was used to investigate sections of the central nervous system and lymphatic tissues from 24 cases of classical scrapie and 25 cases of atypical/Nor98 scrapie in sheep and four healthy control sheep. The PET blot detected deposits of pathological prion protein (PrP^{Sc}) in the brain tissue of all 49 sheep with scrapie but no PrP^{Sc} labelling could be detected in the control sheep. By contrast, not all the atypical/Nor98 scrapie cases were detectable by immuno-histochemistry. The high sensitivity of the PET blot method made it possible to observe that in some atypical/Nor98 cases, deposits of PrP^{Sc} may be restricted to supratentorial brain structures and that the diagnosis may be missed when only testing the obex area, where deposits are common in classical scrapie, and the cerebellar structures, where deposits are considered to be common in atypical/Nor98 cases.

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal neurodegenerative disorders that include, among others, bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease in human beings, chronic wasting disease in cervids (CWD), and scrapie in sheep and goats. The first written evidence of scrapie can be found as early as 1732 (McGowan 1922) and it is therefore the longest-known TSE. The disease-associated agent is thought to be a protease-resistant isoform (PrP^{Sc}) of the so-called prion protein (PrP) (Prusiner 1982), which is encoded by a cellular gene (Oesch and others 1985) and is located on the membrane of cells in various organs, for example, nervous and lymphatic tissues (Bendheim and others 1992). TSEs are characterised by a long incubation period followed by a comparatively short clinical phase and the eponymous spongiform changes in neuropil and neurons observed histopathologically.

Until 2003, scrapie in sheep was commonly thought to be a disease that, once it had occurred in a flock, was transmitted among the animals with susceptible prion protein genotypes. Polymorphisms at codons 136 (V/A), 154 (R/H) and 171 (Q/H/R), resulting in the common alleles VRQ, ARQ, AHQ, ARH and ARR, were shown to affect the susceptibility of sheep to scrapie to a large extent (Belt and others 1995, Hunter 1997). In 2003, an atypical form of scrapie was described and termed Nor98 because it had been diagnosed in Norway from 1998 onwards (Benestad and others 2003). The characteristics of Nor98 scrapie differed from any previously described form with respect to its Western blot profile, epidemiology, the genotypes affected and the pattern of deposition of PrP^{Sc} detected by immunohistochemistry. Reports of similar scrapie cases, often referred to as atypical scrapie, soon followed all over Europe (Buschmann and others 2004a, 2004b, De Bosschere and others 2004, Gavier-Widen and others 2004, Onnasch and others 2004, Orge and others

2004, Everest and others 2006, Konold and others 2006, Nentwig and others 2007, Dagleish and others 2008), the Falkland Islands (Epstein and others 2005) and North America (Cook 2007). In contrast to classical scrapie, sheep with an AHQ (Lühken and others 2004) and/or ARF₁₄₁Q allele (Moum and others 2005) proved to be more susceptible to atypical/Nor98 scrapie, and genotypes with the ARR allele were also affected (EFSA 2005, Buschmann and others 2004b, Orge and others 2004). The retrospective diagnosis of a sheep in the UK with Nor98 in 1989 indicates that the disease had probably been present for a longer time (Bruce and others 2007) but might have been overlooked, possibly due to its occurrence as single cases in flocks (Lühken and others 2007, Benestad and others 2008), the low levels of PrP^{Sc} in the obex region (the standard region used for scrapie diagnosis) and its comparatively low resistance towards proteinase K (Buschmann and others 2004a), which is involved in many diagnostic procedures for recognising PrP^{Sc}.

In general, PrP^{Sc} can be detected using suitable commercially available ELISA kits, but to differentiate between the classical and atypical/ Nor98 forms of scrapie other methods such as Western blot or immunohistochemistry are required. To ensure the detection of atypical/ Nor98 scrapie by these methods, the choice of antibody (Gretzschel and others 2006), the tissue treatment, for example, by proteases, detergents and denaturants, its fixation time in formalin (Privat and others 2000), and not least the choice of brain sections are of great importance.

This paper describes the highly sensitive and specific topographical detection of disease-associated PrP in both classical and atypical/Nor98 scrapie by the paraffin-embedded tissue (PET) blot method.

Materials and methods

Sheep material

Sections were cut from the central nervous system (CNS) and lymphatic tissues (tonsils and/or lymph nodes) of 49 scrapie-positive sheep and four clinically healthy sheep from scrapie-free flocks. The scrapie-positive group consisted of 19 German and five Norwegian sheep diagnosed with classical scrapie either by Western blot or immuno-histochemistry analysis of brain tissue and/or of a tonsil biopsy, and one German and 24 Norwegian Nor98 cases that all displayed the characteristic small molecular fragment of approximately 12 kDa in Western blot analysis; their genotypes and breeds are listed in Table 1. All the Norwegian and German atypical/Nor98 scrapie sheep were single cases from different flocks, but the German classical cases came from three flocks and the Norwegian classical cases came from four flocks, all of which had more than one case of classical scrapie.

Cases	Genotype	Number	Breed
Classical scrapie cases	ARQ/ARQ	15	2 German Merino 2 Blackheaded Mutton 6 German Merino/Blackheaded Mutton crossbreeds 1 Texel/Mountain sheep crossbreed 4 Blackheaded Mutton/Mountain sheep crossbreeds
	VRQ/ARQ	6	2 Texel 1 Texel/Mountain sheep crossbreed 2 Steigar sheep 1 Norwegian pelt sheep
	VRQ/ARH	3	2 Texel 1 Steigar sheep
Atypical/Nor98 scrapie cases	ARQ/ARQ	1	Spæl sheep
	ARQ/AHQ	2	1 German Merino 1 Suffolk/Rygja/Steigar crossbreed
	AHQ/AHQ	6	4 Spæl sheep 1 Steigar sheep 1 Norwegian white sheep
	AHQ/ARH	1	Norwegian white sheep
	AHQ/AFRQ	2	1 Steigar sheep 1 Dala sheep
	AFRQ/AFRQ	5	2 Dala sheep 2 Norwegian white sheep 1 Rygja/Dala crossbreed
	AHQ/ARR	4	1 Norwegian white sheep 2 Dala sheep Spæl sheep
	ARR/AFRQ	2	Norwegian white sheep
	ARR/ARR	1	Steigar
	unknown	1	Rygja/Dala crossbreed
Control cases	ARR/ARR	2	1 German Merino 1 Leine
	ARR/ARH	1	Leine
	unknown	1	Blackheaded Mutton

Table 1: Prion protein genotypes and breeds of 24 cases of classical scrapie, 25 cases of atypical/Nor98 scrapie and four healthy sheep that were investigated

Histopathology

Samples of CNS tissue and lymphoid tissue were fixed in 4 per cent buffered formaldehyde (usually for approximately one week) and embedded in paraffin. Most of the German tissue blocks were also decontaminated with 98% formic acid, followed by another 12 to 24 hours of fixation in 4% formaldehyde before they were embedded. Sections were collected on glass slides and stained with haematoxylin and

eosin or Luxol Fast Blue and periodic acid-Schiff reagent (LFB/PAS) for orientation and discrimination of neuronal nuclei and neural tracts. For the CNS sections, the obex region, midbrain, cerebellum and different cortical regions were examined, and if available, further sections such as the spinal cord, basal ganglia and thalamus were also examined.

PET blot

The PET blot was performed as described in cattle and human beings by Schulz-Schaeffer and others (2000a, b). In brief, 1 to 3 μm sections of paraffin-embedded tissues were cut, placed on 0.45 μm nitrocellulose membranes (Bio-Rad) and dried for two days at 55°C. The membranes were deparaffinised, rehydrated stepwise and then treated with 250 $\mu\text{g}/\text{ml}$ proteinase K (Sigma-Aldrich) overnight at 55°C in proteinase K buffer (10mM Tris-HCl, pH 7.8, 100mM NaCl and 0.1% Brij). After washing the membranes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the proteins were denatured in 4M guanidine thiocyanate for 30 minutes and the membranes were washed again. Immuno-detection was performed after preincubation for 45 minutes in 0.2% casein in PBS containing 0.1% Tween 20 (PBST). The primary antibodies used were the monoclonal antibodies (mAb) L42, P4 (R-Biopharm) and F89/160.1.5 (VMRD), each at a dilution of 1:5000 in TBST for an incubation time of 90 minutes. After washing in TBST, an alkaline phosphatase-coupled goat anti-mouse antibody (D0486; Dako) diluted 1:1000 in TBST was applied for 60 minutes. After thorough washing with TBST, the pH was adjusted by rinsing the blots twice with NTM (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM MgCl_2). The antibody reaction was visualised by the formazan reaction using NBT/BCIP. The blots were dried and evaluated under a dissection microscope.

Immunohistochemistry

The Norwegian scrapie cases were all stained according to the standard protocol (slightly modified from Benestad and others, 2003) at the National Veterinary Institute of Oslo. In brief, the pre-treatment consisted of immersion in formic acid (98%) for 15 minutes followed by hydrated autoclaving at 121°C in 0.01M citric acid (pH 6.1) for 30 minutes. A mild pK digestion (4 $\mu\text{g}/\text{ml}$) at 37°C for five minutes was performed and the sections were incubated overnight at 4°C with mAb F89/160.1.5 (VMRD), raised against residues 142 to 145 of ovine PrP (O'Rourke and others 1998) at a dilution of 1:2000 alone

and together with the mAb 2G11 (1:200). For weakly stained sections, additional antibodies were used [mAb F99/97.6.1 (VMRD), R145 (Jan Langeveld, IDLO, Netherlands), SAF84 and 8G8 (J. Grassi, CEA, France)]. A commercially available kit (K4005, Envision AEC, Dako) was used to enhance the immunolabelling.

In addition, the German cases, the control sheep and selected tissue blocks of each Norwegian scrapie case were immunostained in Goettingen using a different protocol as following.

Tissue sections (1-3 μm) were incubated with pK (50 $\mu\text{g}/\text{ml}$, Sigma-Aldrich) in pK-buffer (10mM Tris pH 7.8; 100mM NaCl; 0.1% Brij) for 15 minutes at room temperature. Slides were treated seven times for three minutes with 10mM citric acid (pH 6) in the microwave (700 W) and incubated with guanidine thiocyanate (4 M) for 15 minutes. Blocking steps with 0.1% H_2O_2 and 0.2% casein in PBST were performed before slides were incubated with the respective primary antibody (mAb P4, L42 or F89/160.1.5) 1:500 in 0.02 % casein/TBS for 90 minutes. The secondary antibody (D0486, Dako, Denmark) was applied 1:500 in TBS for 60 minutes. In-between all steps slides were thoroughly rinsed with TBS. The antibody reaction became visible upon incubation with neo-fuchsin chromogen as substrate and slides were lightly counterstained with haematoxylin.

Results

The three mAbs (P4, L42 and F89/160.1.5) revealed identical deposition patterns of PrP^{Sc} in affected structures of the classical and atypical/Nor98 scrapie cases in PET blots and immunohistochemistry, independently of the protocol used. Nevertheless, variations in the intensity of the immunostaining were recorded, especially with the PET blot method. The mAb P4 provided the most sensitive PrP^{Sc} detection in the atypical/Nor98 scrapie cases and became the antibody of choice for this method, whereas with immunohistochemistry, mAb F89 gave the best results. No PrP^{Sc} deposition was visible in the brain and lymphatic tissue sections of

Figure 1: Obex region of (A) a normal healthy sheep, (B) an early case of classical scrapie, (C) an advanced case of classical scrapie, (D) a case of atypical/Nor98 scrapie analysed by the paraffin-embedded tissue blot method using monoclonal antibody P4. Bar=1 mm. Immunohistochemistry shows in (E) detail of the dorsal motor nucleus of the vagus nerve marked with an arrow in (B) and in (F) labelling in the spinal trigeminal nucleus of the atypical/Nor98 case marked with an arrow in (D). Bar=50 µm

the four healthy control sheep (Fig 1A). With the PET blot, PrP^{Sc} immunostaining was visible in the brain tissue of all the classical and atypical/Nor98 scrapie sheep, but one case of atypical/Nor98 scrapie was consistently negative by immunohistochemistry (Fig 2A).

The following results refer to the PET blot unless indicated otherwise: the deposition patterns and distribution of PrP^{Sc} in the brain corresponded basically with those described for immunohistochemistry in classical scrapie (van Keulen and others 1995) and atypical/Nor98 scrapie (Benestad and others 2008). In one classical case, discrete accumulations of PrP^{Sc} in the dorsal motor

nucleus of the vagus nerve (DMNV) and the solitary tract nucleus were visible by immunohistochemistry and stronger staining was obtained by the PET blot method (Figs 1B, 1E). This case was therefore considered to be an early classical case (van Keulen and others 2008). The other classical cases generally had intense staining of the brainstem and especially of the DMNV (Fig 1C). In contrast, with the PET blot no deposits of PrP^{Sc} were detected in the DMNV of the atypical/Nor98 scrapie cases (Fig 1D). PrP^{Sc} accumulation in the obex of the atypical/Nor98 scrapie cases was often found in the spinal trigeminal nucleus (Figs 1D, 1F), and granules of PrP^{Sc} were frequently present in

Figure 2: Cortex of a three-year-old Dala sheep (AFRQ/AFRQ) that was consistently negative by immunohistochemistry using different protocols and a panel of monoclonal antibodies (mAbs), as in (A) with mAb P4, but gave positive signalling with the PET blot and the same mAb (B). Bar=500 μ m

the white matter, especially in the pyramidal and cerebellar tracts. This distribution corresponds with previous reports (Benestad and others 2008). Among the 15 atypical/Nor98 scrapie cases from which brainstem material was available, only one case had no PrP^{Sc} in the obex region. Sections of spinal cord were available from some of the atypical/Nor98 scrapie sheep, and several of them showed discrete signals in the substantia gelatinosa and in the dorsolateral tract. The substantia gelatinosa and marginal cells were also the most intensely stained structures in spinal cord samples from advanced cases of classical scrapie, but in contrast with the atypical/ Nor98 scrapie cases, all the grey and white matter structures were affected, including sympathetic chain ganglia. The cerebellar cortex in the atypical/Nor98 sheep usually had intense deposits of PrP^{Sc}. In the sheep with no PrP^{Sc} in the obex, only a faint local signal in the granular layer of the cerebellum was observed, which could easily have been

missed. This case was analysed by immunohistochemistry using different protocols and a panel of mAbs (P4, L42, F89, F99, 2G11, 8G8 and R145), but was repeatedly found to be negative in all the brain sections. In contrast, marked PrP^{Sc} labelling was visible in the cerebral cortex (Fig 2B), and there was less intense staining in the basal ganglia and thalamus with the PET blot using mAb P4.

In all the classical cases of scrapie, positive staining was visible in lymphatic tissues (in two cases, no lymphatic tissue was available), as shown for a tonsil in Fig 3. However, no PrP^{Sc} deposits could be found in the lymphatic tissues of any of the 16 atypical/Nor98 sheep for which lymphatic tissues were available.

Discussion

In this study, the PET blot method was applied to sections of CNS and lymphatic tissues of sheep with classical and atypical/Nor98 scrapie. The results support those previously reported for typical and atypical scrapie cases (EFSA 2005), but in addition show that the method is highly sensitive and specific for both types of scrapie. It has already proved to be a useful tool for studies of different TSEs, for example, cattle BSE (Schulz-Schaeffer and others 2000a), BSE in C57Bl/6 mice (Lezmi and others 2006), experimental scrapie in hamsters (McBride and others 2001, Thomzig and others 2004), human TSEs (Schulz-Schaeffer and others 2000b, Peden and others 2006), and for the detection of PrP^{Sc} in the peripheral tissues of sheep diagnosed with classical scrapie (Andréoletti and others 2004, Thomzig and others 2007, Lacroux and others 2007). To the authors' knowledge, this is its first application to tissues of atypical/Nor98 scrapie cases. The high sensitivity of the PET blot method made it possible to observe that in some of the atypical/Nor98 cases, the deposits of PrP^{Sc} may have been restricted to supratentorial (cerebral) brain structures;

Figure 3: Deposits of PrP^{Sc} in the follicles of a tonsil of a sheep with classical scrapie. Paraffin-embedded tissue blot with monoclonal antibody P4. Bar=1 mm

PrP^{Sc} is not necessarily seen in the obex region, as in classical scrapie, and more importantly, may not be visible in cerebellar structures, as is considered to be common for atypical/Nor98 scrapie. The case of a Swiss goat diagnosed with atypical scrapie that had only minimal PrP^{Sc} deposits in the brainstem and cerebellum by immunohistochemistry, but strong PrP^{Sc} labelling in the cerebrum (Nentwig and others 2007), supports the

importance of the present findings with respect to the location sampled for rapid testing. The animal in this study to which this observation refers was only three years old and was the youngest of the Nor98/atypical scrapie cases, suggesting that it might have been an early case of atypical/Nor98 scrapie. It was diagnosed by Western blot, which showed the characteristic Nor98 scrapie profile, and the isolate was also successfully transmitted to tg388 mice (Le Dur and others 2005), but immunohistochemistry did not confirm these results.

The PET blot method can be considered to be a valuable tool for investigating sheep scrapie, because disease-associated PrP deposits can be detected in classical and atypical/Nor98 scrapie cases, even at low levels, so providing the high specificity necessary for diagnosis and research.

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References

- ANDRÉOLETTI, O., SIMON, S., LACROUX, C., MOREL, N., TABOURET, G., CHABERT, A. & OTHERS (2004) PrP^{Sc} accumulation in myocytes from sheep incubating natural scrapie. *Nature Medicine* **10**, 591-593
- BELT, P. B., MUILEMAN, I. H., SCHREUDER, B. E., BOS-DE RUIJTER, J., GIELKENS, A. L. & SMITS, M. A. (1995) Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *Journal of General Virology* **76**, 509-517
- BENDHEIM, P. E., BROWN, H. R., RUDELLI, R. D., SCALA, L. J., GOLLER, N. L., WEN, G. Y., KASCSAK, R. J., CASHMAN, N. R. & BOLTON, D. C. (1992) Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology* **42**, 149-156
- BENESTAD, S. L., ARSAC, J. N., GOLDMANN, W. & NÖREMARK, M. (2008) Atypical/ Nor98 scrapie: properties of the agent, genetics, and epidemiology. *Veterinary Research* **39**, 19
- BENESTAD, S. L., SARRADIN, P., THU, B., SCHÖNHEIT, J., TRANULIS, M. A. & BRATBERG, B. (2003) Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Veterinary Record* **153**, 202-208
- BRUCE, M. E., NONNO, R., FOSTER, J., GOLDMANN, W., DI BARI, M., ESPOSITO, E., BENESTAD, S. L., HUNTER, N. & AGRIMI, U. (2007) Nor98-like sheep scrapie in the United Kingdom in 1989. *Veterinary Record* **160**, 665-666
- BUSCHMANN, A., BIACABE, A. G., ZIEGLER, U., BENCSIK, A., MADEC, J. Y., ERHARDT, G., LÜHKEN, G., BARON, T. & GROSCHUP, M. H. (2004a) Atypical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests. *Journal of Virological Methods* **117**, 27-36
- BUSCHMANN, A., LÜHKEN, G., SCHULTZ, J., ERHARDT, G. & GROSCHUP, M. H. (2004b) Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR). *Journal of General Virology* **85**, 2727-2733
- COOK, W. (2007) Nor98-like strain of scrapie found in Wyoming. Wyoming Livestock Board
- DAGLEISH, M. P., RODGER, S. M., SIMMONS, M. M., FINLAYSON, J., BUXTON, D. & CHIANINI, F. (2008) Atypical scrapie in a sheep in Scotland. *Veterinary Record* **162**, 518-519
- DE BOSSCHERE, H., ROELS, S., BENESTAD, S. L. & VANOPDENBOSCH, E. (2004) Scrapie case similar to Nor98 diagnosed in Belgium via active surveillance. *Veterinary Record* **155**, 707-708
- EFSA (2005) Opinion of the Scientific Panel on Biological Hazards on the request from the European Commission on classification of atypical transmissible spongiform

encephalopathy(TSE) cases in small ruminants. *EFSA Journal* **276**, 1-30

EPSTEIN, V., POINTING, S. & HALFACRE, S. (2005) Atypical scrapie in the Falkland Islands. *Veterinary Record* **157**, 667-668

EVEREST, S. J., THORNE, L., BARNICLE, D. A., EDWARDS, J. C., ELLIOTT, H., JACKMAN, R. & HOPE, J. (2006) Atypical prion protein in sheep brain collected during the British scrapie-surveillance programme. *Journal of General Virology* **87**, 471-477

GAVIER-WIDÉN, D., NÖREMARK, M., BENESTAD, S., SIMMONS, M., RENSTRÖM, L., BRATBERG, B., ELVANDER, M. & AF SEGERSTAD, C. H. (2004) Recognition of the Nor98 variant of scrapie in the Swedish sheep population. *Journal of Veterinary Diagnostic Investigation* **16**, 562-567

GRETZSCHEL, A., BUSCHMANN, A., LANGEVELD, J. & GROSCHUP, M. H. (2006) Immunological characterization of abnormal prion protein from atypical scrapie cases in sheep using a panel of monoclonal antibodies. *Journal of General Virology* **87**, 3715-3722

HUNTER, N. (1997) PrP genetics in sheep and the applications for scrapie and BSE. *Trends in Microbiology* **5**, 331-334

KONOLD, T., DAVIS, A., BONE, G. E., SIMMONS, M. M., KAHN, J., BLAKE-DYKE, M. C., BRACEGIRDLE, J. & SHIMWELL, C. J. (2006) Atypical scrapie cases in the UK. *Veterinary Record* **158**, 280

LACROUX, C., CORBIÈRE, F., TABOURET, G., LUGAN, S., COSTES, P., MATHEY, J., DELMAS, J. M., WEISBECKER, J. L., FOUCRAS, G., CASSARD, H., ELSÉN, J. M., SCHELCHER, F. & ANDRÉOLETTI, O. (2007) Dynamics and genetics of PrPSc placental accumulation in sheep. *Journal of General Virology* **88**, 1056-1061

LE DUR, A., BÉRINGUE, V., ANDRÉOLETTI, O., REINE, F., LAÏ, T. L., BARON, T., BRATBERG, B., VILOTTE, J. L., SARRADIN, P., BENESTAD, S. L. & LAUDE, H. (2005) A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16031-16036

LEZMI, S., BENCSIK, A. & BARON, T. (2006) PET-blot analysis contributes to BSE strain recognition in C57Bl/6 mice. *Journal of Histochemistry and Cytochemistry* **54**, 1087-1094

LÜHKEN, G., BUSCHMANN, A., BRANDT, H., EIDEN, M., GROSCHUP, M. H. & ERHARDT, G. (2007) Epidemiological and genetical differences between classical and atypical scrapie cases. *Veterinary Research* **38**, 65-80

LÜHKEN, G., BUSCHMANN, A., GROSCHUP, M. H. & ERHARDT, G. (2004) Prion protein allele A136 H154Q171 is associated with high susceptibility to scrapie in purebred and crossbred German Merinoland sheep. *Archives of Virology* **149**, 1571-1580

MCBRIDE, P. A., SCHULZ-SCHAEFFER, W. J., DONALDSON, M., BRUCE, M., DIRINGER, H., KRETZSCHMAR, H. A. & BEEKES, M. (2001) Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. *Journal of Virology* **75**, 9320-9327

MCGOWAN, J. P. (1922) Scrapie in sheep. *Scottish*

Journal of Agriculture **5**, 365-375

MOUM, T., OLSAKER, I., HOPP, P., MOLDAL, T., VALHEIM, M., MOUM, T. & BENESTAD, S. L. (2005) Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases. *Journal of General Virology* **86**, 231-235

NENTWIG, A., OEVERMANN, A., HEIM, D., BOTTERON, C., ZELLWEGER, K., DRÖGEMÜLLER, C., ZURBRIGGEN, A. & SEUBERLICH, T. (2007) Diversity in neuro-anatomical distribution of abnormal prion protein in atypical scrapie. *PLoS Pathogens* **3**, e82.doi:10.1371/journal.ppat.0030082

OESCH, B., WESTAWAY, D., WÄLCHLI, M., MCKINLEY, M. P., KENT, S. B., AEBERSOLD & OTHERS (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell* **40**, 735-746

ONNASCH, H., GUNN, H. M., BRADSHAW, B. J., BENESTAD, S. L. & BASSETT, H. F. (2004) Two Irish cases of scrapie resembling Nor98. *Veterinary Record* **155**, 636-637

ORGE, L., GALO, A., MACHADO, C., LIMA, C., OCHOA, C., SILVA, J., RAMOS, M. & SIMAS, J. P. (2004) Identification of putative atypical scrapie in sheep in Portugal. *Journal of General Virology* **85**, 3487-3491

PEDEN, A. H., RITCHIE, D. L., HEAD, M. W. & IRONSIDE, J. W. (2006) Detection and localization of PrPSc in the skeletal muscle of patients with variant, iatrogenic, and sporadic forms of Creutzfeldt-Jakob disease. *American Journal of Pathology* **168**, 927-935

PRIVAT, N., SAZDOVITCH, V., SEILHEAN, D., LAPLANCHE, J. L. & HAUW, J. J. (2000) PrP immunohistochemistry: different protocols, including a procedure for long formalin fixation, and a proposed schematic classification for deposits in sporadic Creutzfeldt-Jakob disease. *Microscopy Research and Technique* **50**, 26-31

PRUSINER, S. B. (1982) Novel proteinaceous infectious particles cause scrapie. *Science* **216**, 136-144

SCHULZ-SCHAEFFER, W. J., FATZER, R., VANDELDELDE, M. & KRETZSCHMAR, H. A. (2000a) Detection of PrPSc in subclinical BSE with the paraffin-embedded tissue (PET) blot. *Archives of Virology* **16**, 173-180

SCHULZ-SCHAEFFER, W. J., TSCHÖKE, S., KRANEFUSS, N., DRÖSE, W., HAUSEREITNER, D., GIESE, A., GROSCHUP, M. H. & KRETZSCHMAR, H. A. (2000b) The paraffin-embedded tissue blot detects PrPSc early in the incubation time in prion diseases. *American Journal of Pathology* **156**, 51-56

THOMZIG, A., SCHULZ-SCHAEFFER, W., KRATZEL, C., MAI, J. & BEEKES, M. (2004) Preclinical deposition of pathological prion protein PrPSc in muscles of hamsters orally exposed to scrapie. *Journal of Clinical Investigation* **113**, 1465-1472

THOMZIG, A., SCHULZ-SCHAEFFER, W. J., WREDE, A., WEMHEUER, W., BRENIG, B., KRATZEL, C., LEMMER, K. & BEEKES, M. (2007) Accumulation of pathological prion protein PrPSc in the skin of animals with experimental and natural scrapie. *PLoS Pathogens* **3**, e66.doi: 10.1371/journal.ppat.0030066

VAN KEULEN, L. J., BOSSERS, A. & VAN ZIJDERVELD, F. (2008) TSE pathogenesis in cattle and sheep. *Veterinary Research* **39**, 24

VAN KEULEN, L. J., SCHREUDER, B. E. C., MELOEN, R. H., POELEN-VAN DEN BERG, M., MOOIJ-HARKES, G., VROMANS, M. E. W. & LANGEVELD, J. P. M.

(1995) Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Veterinary Pathology* **32**, 299-308