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

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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 PrPSc in the brain tissue of all 49 sheep with scrapie but no PrPSc labelling could be detected in the control sheep. By contrast, not all the atypical/Nor98 scrapie cases were detectable by immunohistochemistry. The high sensitivity of the PET blot method made it possible to observe that in some atypical/Nor98 cases, deposits of PrPSc 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.

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, 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 (PrPSc) 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 detection of classical and atypical/Nor98 scrapie by the paraffin-embedded tissue blot method

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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

| Cases          | Genotype | Number of cases | Breed                                      |
|---------------|----------|-----------------|--------------------------------------------|
| Classical      | ARQ/ARQ  | 15              | 2 German merino                            |
|                |          |                 | 2 Blackheaded mutton                       |
|                |          |                 | 2 German merino/blackheaded mutton          |
|                |          |                 | 2 Blackheaded mutton/mountain sheep         |
|                | VRQ/ARQ  | 6               | 2 Texel                                    |
|                |          |                 | 1 Texel/mountain sheep crossbreed           |
|                | VRQ/ARH  | 3               | 2 Texel                                    |
|                |          |                 | 1 Steigar sheep                            |
|                |          |                 | 1 Norwegian pelt sheep                     |
| Atypical/Nor98 | ARQ/ARQ  | 1               | 1 Spel sheep                               |
|                | ARQ/AHQ  | 2               | 1 German merino                            |
|                | AHQ/AHQ  | 6               | 4 Spel sheep                               |
|                | AHQ/ARH  | 1               | 1 Steigar sheep                            |
|                | AHQ/AFRQ | 2               | 1 Norwegian white sheep                    |
|                | AFRQ/AFRQ| 5               | 1 Dala sheep                               |
|                |          |                 | 2 Dala sheep                               |
|                | AHQ/ARR  | 4               | 1 Norwegian white sheep                    |
|                |          |                 | 1 Rygja/Dala crossbreed                    |
|                | ARR/AFRQ | 2               | 2 Norwegian white sheep                    |
|                | ARR/ARR  | 1               | 2 Dala sheep                               |
|                |          |                 | 1 Steigar                                 |
|                |          |                 | 1 Spel sheep                               |
|                | Unknown  | 1               | 1 Norwegian white sheep                    |
|                | ARR/ARR  | 2               | 1 German merino                            |
|                |          |                 | 1 Leine                                    |
|                | ARR/ARH  | 1               | 1 Leine                                    |
|                | Unknown  | 1               | 1 Blackheaded mutton                       |

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**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 immunohistochemistry 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.

**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 per cent formic acid, followed by another 12 to 24 hours of fixa-
tion in 4 per cent 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 (Biollad) and dried for two days at 55°C. The membranes were deparaffinised, rehydrated stepwise and then treated with 250 µg/ml proteinase K (Sigma-Aldrich) overnight at 55°C in proteinase K buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl and 0.1 per cent Brij). After washing the membranes with Tris-buffered saline containing 0.1 per cent Tween 20 per cent Tween 20 (TBST), the proteins were denatured in 4 M guanidine thiocyanate for 30 minutes and the membranes were washed again. Immunodetection was performed after preincubation for 45 minutes in 0.2 per cent casein in PBS containing 0.1 per cent 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 1:200. For weakly stained sections, the additional antibodies mAb F99/97.6.1 (VMRD), R145 (Jan Langeveld, IDLO), SAFA84 and S88 (J. Grassi, CEA, France) were used. A commercially available kit (K4005, Envision ABC; 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 Göttingen by the following protocol.

Tissue sections (1 to 3 µm) were incubated with 50 µg/ml proteinase K (Sigma-Aldrich) in pH-buffer (100 mM Tris-HCl, pH 7.8, 100 mM NaCl and 0.1 per cent Brij) for 15 minutes at room temperature. The slides were treated seven times for three minutes with 10 mM citric acid (pH 6) in a microwave oven at 700 W and incubated with 4 M guanidine thiocyanate for 15 minutes. Blocking steps with 0.1 per cent hydrogen peroxide and 0.2 per cent casein in PBST were performed before slides were incubated with the respective primary mAb (P4, L42 or F89/160.1.5) diluted 1:500 in 0.02 per cent casein/TBS for 90 minutes. The secondary antibody (D0486; Dako) was applied at a dilution of 1:500 in TBS for 60 minutes. Between each step, the slides were rinsed thoroughly with TBS. The antibody reaction became visible upon incubation with neofuchsin chromogen as substrate and the slides were lightly counterstained with haematoxylin.

Results
The three mAbs (P4, L42 and F89/160.1.5) revealed identical deposition patterns of PrPsc 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 P4 monoclonal antibody provided the most sensitive PrPsc 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 PrPsc deposition was visible in the brain and lymphatic tissue sections of the four healthy control sheep (Fig 1a). With the PET blot, PrPsc 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 PrPsc 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 PrPsc 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 PrPsc were detected in the DMNV of the atypical/Nor98 scrapie cases (Fig 1d). PrPsc accumulation in the obex of the atypical/Nor98 scrapie cases was often found in the spinal trigeminal nucleus (Figs 1d, 1f), and granules of PrPsc were frequently present.
in 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 PrPSc 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 PrPSc. In the sheep with no PrPSc 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, S8G and R145), but was repeatedly found to be negative in all the brain sections. In contrast, marked PrPSc 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 PrPSc 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 (Zemzi 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 PrPSc in the peripheral tissues of sheep diagnosed with classical scrapie (Andreoletti 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 PrPSc may have been restricted to supratentorial (cerebral) brain structures; PrPSc 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 PrPSc deposits in the brainstem and cerebellum by immunohistochemistry, but strong PrPSc 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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