Identification of putative atypical scrapie in sheep in Portugal

Leonor Orge,1,2 Alexandre Galo,1 Carla Machado,1 Carla Lima,1 Cristina Ochoa,1 João Silva,1 Manuel Ramos1 and J. Pedro Simas2,3

1Laboratório Nacional de Investigação Veterinária, Lisboa, Portugal
2Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, 2780-156 Oeiras, Portugal
3Laboratório de Microbiologia, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

Experimental transmission of bovine spongiform encephalopathy to sheep has prompted the implementation of a surveillance plan of scrapie in small ruminants by the European Union in all member states. Since its start over 30,000 animals have been tested, and the first seven cases of sheep with detectable PrPres deposition in the central nervous system have been identified in Portugal. Notably, the pattern of PrPres distribution in the brainstem was different from that previously described for scrapie and consistent in all seven animals. Moreover, the profile of the electrophoretic mobility of PrPres after proteinase K treatment was equivalent in all cases analysed but distinct from that observed for scrapie. Notably, four animals had genotypes rarely associated with scrapie, including one animal homozygous for A136R154R171. There were no cases found to exhibit vacuolation, a pattern of PrPres distribution or PrPres electrophoretic mobility corresponding to scrapie. These data reveal a putative atypical scrapie strain in Portugal not linked to specific Prnp genotypes.

Scrapie affects sheep and goats and is the most common natural form of a group of diseases designated transmissible spongiform encephalopathies, which include Creutzfeldt–Jakob disease (CJD) of humans and bovine spongiform encephalopathy (BSE). Scrapie is endemic in several European Union (EU) countries and has been recognized, as a disease identity, for over 250 years (McGowan, 1922). To date, there are no apparent clinical or epidemiological data linking scrapie to human disease. However, since it has been shown that sheep could be experimentally infected with BSE (Foster et al., 1993), the possibility has been raised that BSE could have been accidentally introduced in this species. This would constitute a grave problem in terms of public health, since, in contrast to scrapie, BSE has been directly linked with a new form of CJD (Bruce et al., 1997; Collinge et al., 1996). Furthermore, it has been proposed that scrapie, BSE in sheep could be naturally transmitted and thus become endemic (Foster et al., 2001). Clinically both diseases are indistinguishable (Foster et al., 2001). These possibilities prompted the EU to implement a surveillance scheme for scrapie in small ruminants.

In Portugal, despite the completion of previous scrapie surveillance plans, both at the clinical and histopathological levels, as yet no scrapie cases have been diagnosed. The absence of scrapie in Portugal cannot be attributed to a genetic basis and the possibility has been suggested for subclinical or other forms of scrapie strains to be present (Orge et al., 2003). In this study, as a result of the EU active scrapie surveillance plan, we have identified the first cases of sheep with detectable PrPres deposition in the central nervous system (CNS) in Portugal.

Since its implementation in 2002 (according to EU regulation 999/2001 and subsequent alterations) until February 2004, a total of 30,269 sheep over 18 months of age were screened for the presence of PrPres in the brainstem by the Bio-Rad rapid test done according to manufacturer’s instructions. All the cases that tested positive by the rapid test had been selected for human consumption. These cases were analysed further for the presence of vacuolation by histopathology, for the deposition of PrPres by immunohistochemistry and for the analysis of the PrPres electrophoretic profile by Western immunoblotting, and the Prnp (codons 136, 154 and 171) genotypes were also determined. Brainstems were divided sagittally with one half frozen for rapid test analysis, Western immunoblotting and DNA extraction, and the other half fixed in 10% saline formalin and processed for routine paraffin histology. For immunohistochemistry, three anti-PrP monoclonal antibodies, 2G11 (Institute Pourquier-Farmoquil), SAF84 (SPI-bio) and L42 (RIDA; R-Biopharm), were used in three confirmatory independent assays essentially as previously described (Orge et al., 2000). For 2G11, sections were pretreated with formic acid followed by autoclaving. In the case of SAF84 and L42, sections were, in addition to formic acid treatment, pretreated with proteinase K as described previously (Bencsik et al., 2001; Hardt et al., 2000).
The specificity of PrP<sup>res</sup> immunolabelling was controlled using sections from known scrapie-positive [kindly supplied by the Veterinary Laboratory Agency (VLA) archive] and scrapie-negative cases. For Western immunoblotting analysis of PrP<sup>res</sup>, frozen brainstem tissue was processed according to a new protocol from Bio-Rad developed for ovine tissues. For Prnp genotyping, genomic DNA extracted from frozen brainstem tissue was subjected to PCR with Prnp specific primers (upper and lower primers: 321–340 and 572–590 nt, respectively, according to GenBank accession no. AF180389) to yield an amplicon that encompassed codons 136, 154 and 171. PCR products were then submitted to automated cycle sequencing in both orientations using an ABI Prism 377 DNA sequencer (Perkin Elmer).

As a result of the rapid test screening, seven sheep slaughtered for human consumption that tested positive for the presence of PrP<sup>res</sup> (Table 1) were confirmed for the deposition of PrP<sup>res</sup> in the brainstem at the level of the obex, both by us and the VLA despite the absence of obvious vacuolation. All cases showed the same granular PrP<sup>res</sup> deposition in the neuropil of the spinal tract nucleus of the trigeminal nerve (STN V) (Fig. 1b, e, h and k). Remarkably, no PrP<sup>res</sup> deposition could be detected in the obex region (Benestad et al., 2003). In three sheep (Table 1; cases 4, 6 and 7), focal granular immunostaining in the neuropil of the nucleus of the solitary tract (NST) was also observed (Fig. 1c, f and i). This distribution is unusual because of the consistent absence of detectable PrP<sup>res</sup> in the DVN and the deposition in the STN V. In scrapie, it has been extensively reported that the DVN is the first CNS nucleus to show vacuolation and PrP<sup>res</sup> deposition (Andreolletti et al., 2000; Ligios et al., 2002; Ryder et al., 2001; van Keulen et al., 1995; Wood et al., 1997), hence, is the elected nucleus used for routine diagnosis.

Table 1. Summary of atypical scrapie cases identified

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<th>Case</th>
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*For this case the haplotype was derived independently from DNA extracted from brainstem tissue and lymph node tissue.

Thus, the distribution of PrP<sup>res</sup> deposition obtained with three different anti-PrP specific antibodies was atypical in all seven cases reported here. Recently, scrapie cases have been reported that also diverged phenotypically from typical scrapie (Benestad et al., 2003; Buschmann et al., 2004). In particular, in several atypical scrapie cases that have been reported in Norway, PrP<sup>res</sup> deposition was observed in the cerebellum, midbrain and cerebral cortex, but no PrP<sup>res</sup> could be detected in the obex region (Benestad et al., 2003). In these cases, designated Nor98, although inconsistently, PrPres deposition was also detected in the STN V. These findings are consistent with those described here in that no PrP<sup>res</sup> could be detected in the DVN.

We next sought to evaluate the electrophoretic profile of the PrP<sup>res</sup> in the cases reported here. Frozen brainstem tissue was pretreated with proteinase K and subjected to SDS-PAGE followed by immunoblotting for the detection of PrP<sup>res</sup> (Fig. 2a). We have analysed four of seven cases for which we had sufficient frozen brainstem tissue available. This analysis revealed that the profile of the PrP<sup>res</sup> electrophoretic mobility was equivalent in all cases and ranged from 30 to 12 kDa. This result was consistent with the observation that all seven cases presented the same pattern of PrP<sup>res</sup> distribution in the brainstem. Moreover, coherent with a putative atypical form of scrapie, the PrP<sup>res</sup> electrophoretic mobility in all cases analysed ranged from 30 to 12 kDa clearly distinct from that usually observed for scrapie, which ranges from 30 to 19 kDa (Fig. 2b) (Hope et al., 1999) and BSE in experimentally inoculated sheep, which ranges from 29 to 18-4 kDa (Stack et al., 2002). Significantly, the PrPres electrophoretic profile described here was of the same range and pattern as that described for Nor98 (Benestad et al., 2003).

Notably, four of seven cases reported here had genotypes rarely associated with scrapie, including one animal homozygous for ARR (Table 1). Susceptibility to clinically apparent scrapie is determined by polymorphisms in Prnp, particularly at codons 136 (A, V or T), 154 (R or H) and 171 (Q, R, H or K). Genotypes with VRQ and ARQ alleles are linked to a higher susceptibility to scrapie, whereas, genotypes with AHQ and ARR alleles are linked to a lower susceptibility and increased resistance to scrapie (Billinis et al., 2004; Gombojav et al., 2003; Hunter, 1997). Under experimental conditions, polymorphisms in Prnp have also been shown to influence the transmission of BSE to sheep, with the allele ARQ being associated with a shorter incubation period and ARR with increased resistance (Foster et al., 2001). However, successful transmission of BSE by the intracerebral route to homozygous ARR sheep has been reported recently (Houston et al., 2003). As for Nor98 (Benestad et al., 2003) and other recently reported scrapie cases in Europe (Buschmann et al., 2004), the cases reported here can also be considered as atypical. Thus far, all cases of sheep with PrP<sup>res</sup> deposition in the CNS identified in Portugal are from sheep with no apparent clinical disease and came from flocks dispersed throughout Portugal.
Fig. 1. PrP<sup>res</sup> immunohistochemistry in brainstem at the level of the obex. Numbers identify cases as specified in Table 1. No immunostaining specific for PrP<sup>res</sup> detected in the DVN (a, d, g and j). Marked granular PrP<sup>res</sup> deposition in STN V (b, e and h). Sparse granular PrP<sup>res</sup> deposition in STN V (k) and NST (c, f and i). Antibodies used were as follows: 2G11 (case 4, negative and positive controls), SAF84 (case 6), L42 (case 5 and 7). The NST was not available for the positive control section. Specific PrP<sup>res</sup> signal was visualized with the StreptABC-alkaline phosphatase, New Fuchsin system (DAKO) and tissue sections were counterstained with Mayer’s Haematoxylin.
the country. Notably, of the 30,269 sheep tested none showed a pattern of PrP\textsuperscript{res} deposition coherent with typical scrapie. This finding is consistent with the fact that no clinical scrapie has been diagnosed in Portugal. Thus, it is not clear if the cases described in this study represent an endemic form of a prion disease in sheep not previously identified and not linked to any specific Prnp genotypes or represent a recently acquired new form of scrapie.

In order to gain further insight into the epidemiological and public health relevance of this apparent atypical scrapie, it is extremely important to show if it is transmissible and assess if it constitutes a new scrapie strain like Nor98. The occurrence of such a putative atypical scrapie strain, independently of the Prnp genotype, may be of significance for current EU sheep breeding programmes for selection of non-susceptible haplotypes. Furthermore, given the recent BSE epidemic in Portugal (Donnelly et al., 1999), and thus the geographical risk of transmission of BSE into sheep, a possible link to BSE cannot be excluded. Although, we cannot rule out the possibility of adaptation of BSE in sheep following natural transmission, the fact that the PrP\textsuperscript{res} electrophoretic profile described for this putative scrapie strain is distinct from that observed in natural scrapie makes this link less probable.

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References


