Putative emergence of classical scrapie in a background of enzootic atypical scrapie

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Active transmissible spongiform encephalopathy (TSE) surveillance in small ruminants across Europe was implemented in 2002 following the epizootic of bovine spongiform encephalopathy. Here, we report the potential emergence of classical scrapie in Portugal, in a background of enzootic atypical scrapie. Between 2003 and 2008, 375 459 small ruminants were screened in total, with 328 animals confirmed positive for NOR98 atypical scrapie. During this period, the prevalence rate of atypical scrapie for all years combined was 0.0874 % across the country. In this scenario, classical scrapie emerged as a single outbreak in 2008, with 12 identified cases. In contrast to other European countries, where classical scrapie has been enzootic for decades, these data indicate that, in Portugal, atypical scrapie is the predominant form of TSE. The findings reported here will have implications for the control of classical scrapie in Portugal, namely in terms of keeping the country free of enzootic classical scrapie.

Since a new form of scrapie, designated NOR98 (Benestad et al., 2003), was first described in Norway, many other countries in Europe have reported the presence of this atypical scrapie (Buschmann et al., 2004; De Bosschere et al., 2004; Gavier-Widén et al., 2004; Onnasch et al., 2004; Orge et al., 2004; Everest et al., 2006; Nentwig et al., 2007). It was recognized early on that this newly identified prion type was unlikely to be linked to bovine spongiform encephalopathy (BSE) in small ruminants (Buschmann et al., 2004; Moore et al., 2008). A number of outstanding questions nevertheless remain, including transmissibility, potential of cross-species transmission and impact on animal productivity. Although transmissibility was confirmed experimentally (Le Dur et al., 2005; Simmons et al., 2007; Arsac et al., 2009; Espinosa et al., 2009), as yet, no information is available on whether this atypical prion type, like classical scrapie, is sustained by natural transmission in small ruminants. To answer these questions, more studies on atypical scrapie, including epidemiology and pathogenesis, are needed across Europe. Portugal was one such European country to first report atypical scrapie. In a study where over 30 000 small ruminants were tested for transmissible spongiform encephalopathy (TSE), seven cases of atypical scrapie were identified (Orge et al., 2004). Notably, no classical scrapie was observed in this study, consistent with the lack of previously diagnosed cases in this country. This was unexpected, given that several European countries are enzootic for classical scrapie, including neighbouring Spain. In this study, we have extended our early observations and conducted a large study for TSE surveillance across the Portuguese territory.

Between 2003 and 2008, 375 459 small ruminants were screened in total (Fig. 1a); atypical scrapie was identified in 328 Portuguese small ruminants (326 sheep and two goats) (Fig. 1b). This was achieved by active surveillance and combining rapid testing (TeSeE; Bio-Rad), during the full period of testing, with histopathology examination, immunohistochemistry (2G11 anti-PrP mAb raised against ovine PrP peptide sequence 146-R154-R171-182; Institute Pourquier) and Western immunoblotting (TeSeE Western blot; Bio-Rad). All cases were initially identified by using a rapid test from brainstem samples at the level of the obex, and atypical PrP<sub>res</sub> electrophoretic profile was confirmed by Western immunoblotting. Due to autolysis, vacuolar changes and PrP<sub>res</sub> distribution were only analysed for 245 brainstem and 126 cerebellum samples. The overall prevalence in animals tested was 0.0874 % (calculated as number of positive animals/number of animals tested × 100), with fallen stock and healthy slaughter at 0.1126 and 0.0807 %, respectively (Fig. 1c). The observed prevalences were statistically significantly
Atypical scrapie

presented clinical signs that mostly included abnormal gait, were not available. A total of 30 animals retrospectively
surveillance of affected flocks, since a culling policy was
cohabitants, with a mean of one to three cases per flock.

74.44 % were between 5 and 10 years of age and 13.89 %
and pattern as that described for NOR98, showing three to
were between 5 and 10 years of age, 74.44 % were between
were older than 10 years. As a result of intensive
5 years of age, 74.44 % were between 5 and 10 years of age
PrPres deposition was most consistently observed in
characteristics of atypical scrapie, vacuolation and PrPres deposition were not observed in the dorsal vagal nucleus (DVN). Hence, in
terms of diagnostic value, the cerebellum was consistently
positive (Table 1). This result confirms that, in the case of
atypical scrapie, the cerebellum has high diagnostic value,
as suggested previously (Benestad et al., 2003).

The PrPres electrophoretic profile was of the same range
and pattern and as that described for NOR98, showing three
to five bands between 30 and 12 kDa depending on the case
(Fig. 2b). The band-pattern designation was based on that
of Arsac et al. (2007). Combined, our results confirm that
atypical scrapie in Portugal is NOR98. Prnp genotyping
(performe as reported previously; Orge et al., 2004) of
atypical scrapie cases revealed a variety of genotypes, which
included ARR/AFRQ, ARR/ARR, ARQ/AFRQ and AFRQ/
AFRQ (Fig. 2c). This result confirmed the previously
reported association with genotypes rarely linked with
classical scrapie (Orge et al., 2004). It also shows that the
allele AFRQ is represented more than in studies that
reported the AHQ allele as being most frequently
associated with atypical scrapie (McIntyre et al., 2008;
reviewed by Benestad et al., 2008; Fediaevsky et al., 2009).
This could be related to the low frequency of this allele in
the Portuguese sheep population (Orge et al., 2003).
Notably, as a result of this large screening effort, 12 sheep
tested positive for classical scrapie by rapid test, his-
topathology (Fig. 2d), PrPres deposition (Fig. 2e) and
Western blotting for PrPres (Fig. 2f). Neuronal vacuolation
in the DVN and neuropil vacuolation in the ventral border
of the DVN, typical of classical scrapie, were observed. All

Fig. 1. Epidemiological data of atypical scrapie in Portuguese sheep from 2003 to 2008 show that it is enzootic. (a) Distribution
of TSE testing in small ruminants per target group. Testing healthy
slaughter was carried out in all animals over 18 months old since
2004 to 31 August 2008. After this period, the testing was performed as random sampling according to Annex III of
Regulation (EC) no. 999/2001. The number of samples from
fallen stock increased in 2005 due to the implementation of a fallen
stock collection system in the south of the country, and in 2007
when the system was extended to the north. (b) Number of atypical scrapie cases in Portuguese small ruminants detected annually. (c)
Prevalence of atypical scrapie in animals tested per target group. Empty bars, healthy slaughter; filled bars, fallen stock.

different, with a P-value of 0.007 (as assessed by using a
chi-squared test). Age distribution from 180 atypical scrapie cases revealed that 11.67 % were <5 years of age, 74.44 %
were between 5 and 10 years of age and 13.89 % were older than 10 years. As a result of intensive
surveillance of affected flocks, since a culling policy was not implemented, atypical scrapie was detected in 19
cohabitants, with a mean of one to three cases per flock. Data regarding the number of flocks with secondary cases
were not available. A total of 30 animals retrospectively
presented clinical signs that mostly included abnormal gait,
loss of condition and tremors. Of note, all 30 animals had
at least one allele (AFRQ and AHQ) that confers susceptibility
to NOR98, except for three animals that presented an ARR/ARR genotype, which is associated with resistance
to scrapie.

Histopathology examination revealed that most cases did
not show typical vacuolation. However, when vacuolation
was present, it was observed in the neuropil of the spinal
tract of the trigeminal nerve and in the molecular layer
of the cerebellum (data not shown). PrPres deposition,
presenting as punctate to coarse granular deposits, was
observed in both the grey [Fig. 2a(i, ii)] and the white
[Fig. 2a(iii)] matter in the brainstem at the level of the obex
and in the cerebellum. PrPres distribution between cases
was variable, as reported previously (Nentwig et al., 2007;
Benestad et al., 2008), with several combinations observed
(Table 1). It was not possible to correlate variability of
PrPres deposition with factors such as surveillance stream,
genotype, PrPres glycoform profile or clinical signs.
However, PrPres deposition, when present in the brainstem,
was detected consistently in the spinal tract nucleus of
the trigeminal nerve and in the white matter tracts in the obex.
Although less consistently, PrPres deposition was also
observed in the solitary nucleus. In cerebellum, when
available, PrPres deposition was most consistently
observed in the granular layer [Fig. 2a(iv)], followed by the molecular
layer of the cerebellum [Fig. 2a(iv)]. Characteristic of
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in the DVN and neuropil vacuolation in the ventral border
of the DVN, typical of classical scrapie, were observed. All
cases showed intracellular immunolabelling (intraneuronal and intraglial) and the extracellular deposition was particulate/coalescing, perineuronal and linear. In three cases, it was also observed that the extracellular immunolabelling was stellate and perivascular. The vascular type was not observed. Classical scrapie showed a three-band pattern between 30 and 19 kDa. Vacuolation, PrP res deposition types and PrP res glycoform electrophoretic mobility were according to those described for classical scrapie (Wood et al., 1997; Hope et al., 1999; Ryder et al., 2001; Gonzalez et al., 2002).

To distinguish between BSE and scrapie, samples were submitted to the discriminatory test for strain typing (CEA/Bio-Rad) and none of the samples were BSE-like (data not shown). Whilst atypical scrapie was scattered throughout the country, classical scrapie was confined to two nearby dairy flocks, where all animals were bred locally of Portuguese breeds crossed with exotic breeds. One flock with 273 Assaf-crossed sheep tested positive for classical scrapie in six fallen stock and one healthy slaughtered animal. This flock also had an animal with an ARQ/AFRQ genotype from healthy slaughter that tested positive for atypical scrapie NOR98. This entire flock was culled and all of the animals tested negative for PrPres by rapid test. The other flock, with 2343 sheep of crossed Merino Beira Baixa, Lacaune and Assaf breeds, had one animal from healthy slaughter, one fallen stock and three cohabitants that tested positive for classical scrapie. This flock was genotyped for purposes of selective culling. Random testing of culled animals resulted in three cohabitants testing positive for rapid test. At the time of writing this manuscript, this flock had never shown animals with clinical signs of TSE or positive cases by rapid testing screening. Affected animals

![Fig. 2. Characterization of Portuguese atypical and classical scrapie cases. (a) PrP res immunolabelling in the brainstem at the level of the obex (i, iii) and in the cerebellum (ii, iv) of representative atypical cases: (i) STN V; (ii) molecular layer of cerebellum; (iii) white matter; (iv) cerebellum. Specific PrP res signal was visualized with Vectastain–peroxidase, DAB (Vector). All sections were counterstained with Mayer’s haematoxylin. The magnifications used are indicated. (b) PrP res electrophoretic profile after proteinase K treatment in eight representative atypical cases. Upper panel, a four-band pattern is shown in lane 1 and a three-band profile in lanes 3–7. Lane 2 corresponds to a negative control. A five-band profile is represented in lanes 8 and 9 (lower panel). ScC, Positive classical scrapie control, kindly provided by the Veterinary Laboratory Agency (UK) archive; M, molecular mass marker (in kDa). (c) Distribution of Prnp genotype in atypical cases in Portuguese sheep. (d) Neuronal vacuolation in DVN (haematoxylin and eosin). (e) PrP res immunolabelling observed in classical cases: (i) intraneuronal (DVN) and (ii) particulate/coalescing (DVN). DAB (Vector) peroxidase, counterstained with Mayer’s haematoxylin. (f) Western blot analysis of PrP res from classical scrapie cases [lanes 1 and 2 correspond to two cases identified in one flock and lane 3 to another case from the other flock; ScC, positive classical scrapie control, kindly provided by the Veterinary Laboratory Agency (UK) archive]. M, Molecular mass marker (in kDa).]
ranged between 18 and 84 months of age. The most frequent genotype linked with these cases of classical scrapie was ARQ/ARQ (susceptible genotype), with one animal being ARQ/AFRQ (a frequent genotype associated with NOR98 scrapie), one ARH/ARQ and another ARR/AHQ (intermediate and resistant genotypes for classical scrapie, respectively). The latter genotype is unexpected and, as suggested previously (Groschup et al., 2007), indicates that genetic resistance to scrapie is not absolute for resistant genotypes.

Our results confirm the presence of classical scrapie in Portugal against a background of enzootic NOR98 scrapie, independent of Prnp genotype. Whether NOR98 is spontaneous or naturally transmitted remains to be established as yet, even though several case–control studies suggest that NOR98 is spontaneous (Hopp et al., 2006; Luhken et al., 2007; Fediaevsky et al., 2009). The origin of the natural scrapie outbreak does not appear to be linked to the import of animals, as affected animals were bred locally. However, in contrast to other European countries, where classical scrapie has been enzootic for decades, our data indicate that, in Portugal, atypical scrapie is the predominant form of TSE in small ruminants. The findings reported here should be taken as a forewarning of the potential emergence of classical scrapie in Portugal in an atypical scrapie background.

References


Table 1. Variability of PrP<sup>res</sup> immunolabelling and electrophoretic profile observed in atypical scrapie

<table>
<thead>
<tr>
<th>No. cases</th>
<th>PrP&lt;sup&gt;res&lt;/sup&gt; immunolabelling</th>
<th>Western immunoblot profile (no. cases)</th>
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<tr>
<td></td>
<td>Brainstem–obex</td>
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STN V, Spinal tract nucleus of trigeminal nerve; NST, nucleus of the solitary tract; WM, white matter; G, granular layer; M, molecular layer; NA, not available.


