Effect of PrP genotype and route of inoculation on the ability of discriminatory Western blot to distinguish scrapie from sheep bovine spongiform encephalopathy

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Procedures for discriminating scrapie from bovine spongiform encephalopathy (BSE) in sheep are relevant to ascertain whether BSE has entered the sheep population. This study was aimed at investigating whether the suitability of an official EU discriminative method is affected by the sheep PrP genotype and route of infection.

Sheep infected by the oral or intracerebral routes with scrapie, bovine spongiform encephalopathy (BSE) or sheep BSE were included in this study. All BSE samples were discriminated by Istituto Superiore di Sanità Discriminatory Western-Blot (ISSDWB), irrespective of the route of inoculation and genotype. In scrapie, host genotype and route of inoculation did not affect PrPres molecular features. Conversely, a higher molecular variability was observed in sheep BSE, with ARR/ARR sheep showing the lowest L42/P4 ratios and the lowest glycoype and ARQ/AHQ sheep the highest L42/P4 ratios.

Our results suggest that in BSE, but not in scrapie, PrPSc features can be altered by the host PrP genotype, although not enough to hamper BSE recognition by ISSDWB.

Transmissible spongiform encephalopathies (TSEs), including Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goat and BSE in cattle, are fatal neurodegenerative diseases. Scrapie has been endemic in many European countries for 250 years and it is not considered to be zoonotic. BSE was first detected in cattle in 1986 and it has been accidentally transmitted to other species, including humans in the form of variant CJD (Bruce et al., 1994, 1997; Foster et al., 1996; Lasmèzas et al., 2001). It is theoretically possible that BSE has infected the EU sheep flock; thus, providing a secondary source of BSE exposure to humans. The European ovine population has probably been exposed to BSE contaminated meat-and-bone meal up to the feed ban in 2001, with a peak exposure in 1988 (DNV, 2001). This exposure might have caused cases of BSE in sheep (Foster et al., 1993), which in turn could have passed BSE to other sheep via horizontal transmission (Bellworthy et al., 2005), potentially leading to the natural spread of BSE in EU sheep flock. However, to date no evidence for this scenario occurring emerged from the EU surveillance.

TSEs are characterized by the accumulation in the central nervous system of an abnormal isoform (PrPSc) of the cellular prion protein (PrPC). Treatment with protease K (PK) removes some residues from the N-terminal domain of PrPSc, yielding PrPres, which consists of uncleaved, monoglycosylated and diglycosylated PrP fragments. Experimental BSE in sheep is characterized by the accumulation of a distinctive PrPres type, which is discriminated from scrapie PrPres on the basis of molecular mass and glycoform pattern (Hill et al., 1997; Hope et al., 1999; Baron et al., 2000; Nonno et al., 2003). The lower apparent molecular mass of BSE PrPres depends on a more C-terminal PK cleavage site compared with scrapie PrPres and this feature was used for developing discriminatory methods (Stack et al., 2002; Lezmi et al., 2004; Thuring et al., 2004; Gretzschel et al., 2005; Simon et al., 2008; Pirisinu et al., 2011).

Since 2004 the EU implemented a surveillance scheme for BSE in small ruminants (EC reg. 999/2001). The surveillance is based on large-scale testing of small ruminant TSE cases with molecular techniques, which are able to discriminate the majority of scrapie cases from BSE. Samples with BSE-like features are then analysed with alternative molecular techniques and discriminatory immunohistochemistry (Jeffrey et al., 2001). Samples which are still BSE-like at this stage would undergo biological typing in mice for specific
Biochemical variations of PrPSc in sheep BSE

identification of the BSE strain. More than 8000 EU sheep TSE cases have been analysed so far with no definitive BSE cases confirmed to date. The EFSA concluded in 2007 that the prevalence of BSE in sheep may be zero or, if present, very low (EFSA, 2007). Similar conclusions were derived from retrospective surveys in the EU (Gretzschel et al., 2005; Stack et al., 2006, Simon et al., 2008; Vulin et al., 2011). However, discriminatory tools have been validated mainly using ARQ/ARQ samples, while different PrP genotypes, including ARR/ARR, were permissive to experimental BSE infection (Houston et al., 2003). These studies raised the question of our ability to recognize sheep BSE on molecular grounds, as the possible impact of the genotype, the route of infection and the number of passages on the biochemical properties of BSE PrPSc are still poorly understood. We partially addressed these points by analysing the molecular properties of PrPSc in 36 clinically affected Sarda breed sheep experimentally infected with scrapie, cattle BSE or sheep BSE (Vaccari et al., 2007).

Among the scrapie sheep that were inoculated (n=20), eight were orally inoculated (OR) (5×ARQ/ARQ and 3×ARQ/AHQ) and 12 were intracerebrally (IC) inoculated (4×ARQ/ARQ, 3×ARQ/AHQ, 1×ARQ/AHQ, 1×ARQ/ARH, 2×ARH/AHQ and 1×ARQ/ARR) with the same ARQ/ARQ scrapie homogenate. Among the BSE inoculated (n=14), five were orally inoculated (3×ARQ/ARQ and 2×ARQ/AHQ) and nine were IC inoculated (2×ARQ/ARQ, 1×ARQ/ARH, 1×ARQ/AHQ and 5×ARR/ARR) with the same cattle BSE homogenate. We have previously reported the survival time of sheep included in this study (Vaccari et al., 2007), with the exception of the ARQ/ARQ scrapie IC [survival time of 1669 days post-inoculation (p.i.)] and five ARR/ARR BSE IC sheep (1495–1751 days p.i.). The two ARQ/ARQ sheep were IC inoculated with an ARQ/ARQ BSE sheep brain homogenate (443 and 477 days p.i.).

Molecular typing of PrPSc was performed by discriminatory immunoblotting, carried out according to the ISSDWB method described on the discriminatory testing handbook (http://www.defra.gov.uk/vla/science/docs/sci_tse_rl_handbookv4jan10.pdf). The principle of discrimination is based on the differential N-terminal cleavage by PK, revealed by using mAb P4 (R-Biopharm) whose epitope (residues 93–99 of ovine PrP) is partially lost after PK digestion of BSE samples. In the present study, mAb L42 (R-Biopharm), epitope 148–153 of ovine PrP, was used as a PrP-core antibody instead of SAF84, as SAF84 does not bind ARR PrP (unpublished data). The discrimination is obtained by parallel testing of samples with both antibodies and by measuring the apparent molecular mass of the unglycosylated PrPSc with L42 and the ratio between the signal obtained with L42 and P4, relative to the L42/P4 ratio of the scrapie internal control (L42/P4 ratio). Samples with a molecular mass >0.5 kDa lower than the scrapie control and with a L42/P4 relative ratio >2 are considered BSE-like. As an additional discriminatory parameter, the relative proportions of diglycosylated, monoglycosylated and unglycosylated PrPSc fragments (glycoform ratio) were measured in L42 blots. Chemiluminescence was detected with the VersaDoc imaging system (Bio-Rad) and signal quantification was performed with QuantityOne software (Bio-Rad).

Scrapie OR ARQ/ARQ and BSE OR ARQ/ARQ controls were included in each blot (samples A2 and B2 in Supplementary Table S1, available in JGV Online). Samples were analysed in at least three independent experiments. Statistical analyses were carried out by unpaired t-test (two-tailed P-values) for comparing scrapie and BSE groups, as well as IC versus OR sheep within each group, and by one-way ANOVA and Bonferroni’s post-test for analysing the effect of the PrP genotype.

For all scrapie samples, similar PrPSc electrophoretic patterns were observed with L42 and P4, while all BSE samples were poorly recognized by P4 (Fig. 1). Sheep BSE samples were discriminated from scrapie, independently of the PrP genotype or the route of inoculation, showing always a molecular mass >0.5 kDa lower than the scrapie control and a L42/P4 ratio >2. The individual molecular masses and L42/P4 ratios are reported in Supplementary Table S1 and in Fig. 2(a, b). Mean molecular masses (±SD) were 17.91±0.11 for scrapie and 17.10±0.09 for sheep BSE (P<0.0001), while mean relative ratios (±SD) were 1.07±0.12 for scrapie and 16.11±5.66 for sheep BSE (P<0.0001). The glycoform ratio also allowed us to differentiate scrapie and BSE samples (Fig. 2c), with significant differences for diglycosylated (0.47±0.02 in scrapie and 0.64±0.02 in BSE, P<0.0001) and monoglycosylated fragments (0.33±0.01 in scrapie and 0.26±0.01 in BSE, P<0.0001).

Although scrapie and BSE were significantly different for all parameters considered, it was possible to observe some degree of variability within both groups. This was particularly evident when looking at the glycoform ratio of sheep BSE samples, with some BSE samples showing a glycoform ratio more akin to scrapie (Fig. 2d). We thus performed statistical comparisons to see if this variability depended on the route of inoculation and/or the PrP genotype of the affected sheep.

For the scrapie samples, statistical analyses did not show any significant differences with regard to variations in sheep genotype or the route of inoculation. On the contrary, for sheep BSE both, the route of inoculation and the PrP genotype showed some effect.

In sheep BSE, the route of inoculation showed a low but significant difference between the di- and monoglycosylated fragments, with OR sheep showing a higher proportion of diglycosylated PrPSc (P<0.05) and lower proportion of monoglycosylated PrPSc (P<0.05) than IC sheep (Fig. 2d). There were no significant differences for molecular mass or the L42/P4 ratio by the route of inoculation.

Statistical analysis of the effect of the genotype in sheep BSE when the sheep were grouped as ARQ/ARQ, ARQ/AHQ and ARR/ARR showed that there were highly significant differences for the L42/P4 signal ratios (P<0.0001) and
**Fig. 1.** Discriminatory WB in sheep with scrapie and BSE. Representative WBs depicting PrP\(^{\text{res}}\) from sheep inoculated with scrapie or BSE. The IDs of the samples are reported on each lane (see Supplementary Table S1). Replica blots were developed with L42 and P4 mAbs. The molecular mass marker is reported on the right of the blots (kDa). Representative differences among BSE samples are shown: ARR/ARR samples (B10, B11, B13 and B14) were less glycosylated and less well discriminated by P4 than other BSE samples ARQ/AHQ (B9), C1 (ARQ/ARQ\(^{2\text{nd}}\)) was less well discriminated than C2 (ARQ/ARQ\(^{2\text{nd}}\)) by P4 binding.

**Fig. 2.** Molecular features of PrP\(^{\text{res}}\) from sheep with scrapie and BSE. Graphs depicting the molecular mass (a), L42/P4 ratio (b) and glycoform ratio (c) of scrapie (■) and BSE (○) samples (left panels). In (a, b) the dashed line represents the cut-off values for discriminating BSE from scrapie samples. In right panels, the graphs show the glycoform ratio (d and e) and the L42/P4 ratio (f) in sheep with BSE. In (d) OR (○) and IC (■) sheep are compared. In (e) ARR/ARR sheep (○) are compared with other genotypes (■). In (f) ARQ/ARQ (■), ARQ/AHQ (○), ARR/ARR (■) and ARQ/ARQ\(^{2\text{nd}}\) (○) are compared. Symbols represent mean values and error bars represent SD.
the glycoform ratios ($P<0.0001$), but not for the apparent molecular mass.

For the glycoform ratio, there were significant differences for the diglycosylated and monoglycosylated proportions between ARR/ARR and ARQ/ARQ ($P<0.001$) and between ARR/ARR and ARQ/AHQ ($P<0.001$) (Figs 1 and 2c). The mean values ($\pm$SD) for the diglycosylated and the monoglycosylated proportions were $0.67 \pm 0.03$ and $0.23 \pm 0.02$; $0.67 \pm 0.01$ and $0.24 \pm 0.01$ and $0.58 \pm 0.01$ and $0.30 \pm 0.01$ for ARQ/ARQ, ARQ/AHQ and ARR/ARR groups, respectively.

For the L42/P4 ratio, there were significant differences between ARQ/AHQ and ARR/ARR ($P<0.001$), ARQ/ARQ and ARQ/AHQ ($P<0.01$) and between ARR/ARR and ARQ/ARQ ($P<0.05$) (Figs 1 and 2f). The mean values ($\pm$SD) for L42/P4 ratio were $15.95 \pm 4.32$, $25.40 \pm 10.44$ and $11.35 \pm 4.36$ for ARQ/ARQ, ARQ/AHQ and ARR/ARR groups, respectively.

The two sheep that had been inoculated as a second passage of BSE in sheep showed glycoform ratios and molecular masses similar to those observed in ARQ/ARQ sheep inoculated IC with cattle BSE. However, the L42/P4 ratio from one of these two sheep had a ratio lower than expected (Figs 1 and 2f), similar to that observed in ARR/ARR sheep.

The variability of L42/P4 ratio among PrPres from sheep with different genotypes infected with BSE would suggest a different N-terminal cleavage site by PK, although no significant variation of the molecular mass of PrPres was observed. Protease treatment with increasing PK doses showed that the amount of PrPres decreased at similar rates in ARR/ARR and ARQ/AHQ samples, while the P4-positive proportion was higher in ARR/ARR samples (Fig. 3a) at all PK concentrations. Indeed, the L42/P4 ratio was lower in ARR/ARR samples and this difference increased with increasing PK concentrations (Fig. 3b). These results suggest that PK cleavage is slightly more N-terminal in ARR/ARR compared with ARQ/AHQ sheep, irrespective of the PK dose used.

Overall, our results showed that the ability of ISSDWB in discriminating BSE was not impaired by the route of inoculation, the PrP genotype or by passage in sheep. Indeed all sheep BSE samples retained BSE-like properties and were discriminated from scrapie. These data show that ISSDWB is sensitive in discriminating BSE and is suitable for BSE surveillance in sheep. Nonetheless, our results show that in BSE, but not in scrapie, the route of inoculation and the PrP genotype had a significant effect on the molecular features of PrPres.

The route of inoculation only slightly influenced the degree of glycosylation of BSE PrPres, while the PrP genotype accounted for most of the variability observed. Indeed, ARR/ARR sheep showed less pronounced BSE molecular features compared with ARQ/ARQ and ARQ/AHQ sheep, i.e. lower antibody ratio and lower proportion of diglycosylated PrPres. These results are in line with those obtained by differential ELISA (Simon et al., 2008), which showed a lower antibody ratio in BSE-challenged ARR/ARR compared with ARQ/ARQ sheep, and by Western blot analysis of a single ARR/ARR sheep (Ronzon et al., 2006).

The low number of BSE second passage samples studied prevented a statistical assessment of the effect of BSE sheep-to-sheep passage. However, the L42/P4 ratio obtained from one of the two ARQ/ARQ sheep infected with sheep-passaged BSE was lower than expected, being the lowest among ARQ/ARQ and ARQ/AHQ sheep investigated. This is reminiscent of the results obtained on a larger set of samples by Stack and collaborators, who showed a decreasing trend of the 6H4/P4 ratio as the number of BSE passages increases (Stack et al., 2009). Based on these results and considering that sheep-passaged BSE may be more easily transmitted to ARR/ARR sheep than cattle BSE (González et al., 2007), it seems important to continue the monitoring of the molecular features of PrPres in experimental sheep-to-sheep BSE passages, involving sheep of different PrP genotypes to see if successive changes in the

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**Fig. 3.** PK titration of PrPres from ARR/ARR and ARQ/AHQ sheep with BSE. (a) Brain homogenates from ARR/ARR (B10) and ARQ/AHQ (B9) samples were digested with increasing concentrations of PK between 0.0008 and 1.4 mg ml$^{-1}$ and then probed with L42 (upper panel) or P4 (lower panel). (b) Graph showing the L42/P4 absolute ratio in ARR/ARR (●) and ARQ/AHQ (○) samples derived from the WB shown in (a).
molecular features might eventually lead to the loss of the BSE molecular signature.

Our and previous data concur in demonstrating that slight but significant variations in the molecular phenotype are produced in sheep challenged with BSE, according to their PrP genotype and to the number of passages in sheep. It has been previously shown that pathological features able to discriminate between sheep BSE and scrapie, i.e. intracellular antibody labelling patterns (Martín et al., 2005) and the immunohistochemical profile of PrP\textsuperscript{res} accumulation in the brain (González et al., 2005), were not altered by PrP genotype, route of inoculation or sheep-to-sheep passage. However, subtle quali-quantitative differences in the immunohistochemical profile were observed in ARR/ARR sheep (González et al., 2005). The extent to which these molecular and pathological variations actually represent mere phenotypic differences or are the result of a strain shift of BSE upon passage in hosts with a different PrP sequence remains to be established. This could have an impact on the risk for human health, in light of recent developments in the field.

Acknowledgements

This work was supported by the European Union (Neuroprion Network of Excellence) CT-2004-506579 and the Italian Ministry of Health.

References


