Is there any atypical bovine spongiform encephalopathy (BSE) in Belgium? The screening of BSE-positive cattle (1999-2010) aging seven years and older

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The prion diseases

Prion diseases are infectious neurodegenerative diseases with slow development and lethal outcome. The active agents in this disease family are called prions. Prion diseases are unique as a normal host cellular protein, prion protein (PrPc), is usually affected by conformational change and aggregation which leads to the accumulation of PrPd (associated to the disease), usually in the nervous system. There is indeed no specific DNA or RNA involved. PrPd is partly resistant to digestion by proteases and the resultant product of such digestion (PrPres) is used in diagnostic applications as a highly reliable disease marker. This PrPres presents three (glyco)forms: unglycosylated, monoglycosylated and diglycosylated. Usually in clinical cases, the brain shows microscopic symmetrical spongiosis. Prion diseases are therefore also called transmissible spongiform encephalopathies (TSE).

These diseases in animals are mainly transmitted by the dietary route and already described for centuries in sheep as scrapie. Other examples of TSE are chronic wasting disease in deer and elk (CWD), Creutzfeldt-Jakob disease in humans (CJD), transmissible mink encephalopathy (TME) and bovine spongiform encephalopathy (BSE), also known as mad cow disease. Evidence for transmission of BSE was found in various other mammals including humans, where it causes a variant form of CJD (vCJD), which affects especially young persons.

While in scrapie and other TSEs strain variations were found, BSE initially showed strain homogeneity. Indeed, incubation time, vacuolar lesion profile and biochemical signature of PrPd were identical for all investigated cases. This uniform aspect of the BSE prions during the epidemic was probably due to the contamination of the food chain by a single strain in United Kingdom.
Atypical forms of BSE

However, due to the extensive active surveillance and new research techniques, rare variants of BSE have been reported since 2003 of which most could be classified in two new types of BSE: H- and L-type. They were named like that because Western blot studies showed that, in comparison to classical BSE (C-type), these two atypical forms are characterised by a higher or lower molecular mass of the unglycosylated PrP\textsuperscript{Sc} (they were therefore named H- and L-type, Fig. 1). L-type is also called bovine amyloid spongiform encephalopathy or BASE. The PrP\textsuperscript{Sc} glycoprofile is a very practical marker for the L-type, which shows a much lower proportion of diglycosylated PrP\textsuperscript{Sc} (<50% of total PrP\textsuperscript{Sc}) than C-type (>55% of total PrP\textsuperscript{Sc}, Fig. 2). For H-type, a relatively high reactivity with a given antibody (12B2) is archetypal. A second characteristic of H-type involves a dualistic glycoprofile depending on the antibody used (Fig. 2). As the brain area of the PrP\textsuperscript{Sc} deposition can differ drastically between the L- and C-type and potentially also between H- and C-type, it would be better if the sampling techniques presently used are adapted to these atypical types.

Such types are very rare; only 60 cases were described worldwide and these were all detected in old bovines (at least eight years in age), except one case from a 23 month old cow that could not be further investigated in depth due to shortage of sample. They were reported on different continents. Indeed they might correspond to natural “sporadic” forms of BSE, not originating from the contamination of the food chain.

**Fig. 1:** Western blots comparing reference samples of L-, C- and H-type isolates. From top to bottom, the three bands of each sample correspond to di-, mono- and unglycosylated forms of the PrP. The different reaction of C-type in comparison to H-type is visible with 12B2 antibody (a), the higher molecular mass of H-type is revealed by Sha31 antibody (b) and the presence of a fourth band in H-type is shown by SAF84 antibody (c, arrow). With Sha31 antibody, L-type shows a greater proportion of monoglycosylated band compared to the diglycosylated band than C-type. A molecular marker is displayed (MM, three arrow heads represent position of 20, 30 and 40 kDa).
Fig. 2: Glycoprofiles of the 39 resistant prion proteins (PrP\textsuperscript{resist}), obtained with antibody 94B4. The PrP possesses two, one or no carbohydrates attached. These three forms are found in various proportions in a given animal. Colours refer to the percentage of the diglycosylated form. The average glycoprofiles (with standard deviations) obtained with 94B4 for a reference isolate of H-type and L-type are indicated (H\textsubscript{c} and L, showing lower diglycosylated percentage, <50% for L-type, 3 replicates). HB represents the H-type glycoprofile using Sha31 antibody, not allowing a clear discrimination. The three-arrow symbol is to assist the reader in finding the direction of each point to the different axes.
Is there atypical BSE in Belgium?

It is essential for the BSE surveillance programmes to determine the prevalence on a global level of these atypical types. Therefore the Belgian National Reference Laboratory for TSEs retrospectively analysed the bovines of at least 7-years-old in its archive of BSE-diagnosed cattle, using the latter criteria and techniques, in order to determine whether these types of atypical BSE were present in old BSE-positive bovines in Belgium. In the past, a 60 month old unclassified BSE case was described in Belgium when molecular typing techniques and comparison with L- and H-types was not available. This sample was re-examined with the current techniques in this study.

Firstly the H-, L- and C-type reference samples were investigated to confirm the ability of the laboratory to discriminate these types by Western blot showing their glycoprofiles. In the H-type sample, the unglycosylated band migrated at a higher gel position than in the C-type; moreover the H-type reacted strongly to 12B2 antibody contrary to C- and L-type (Fig. 1). It showed a dualistic glycoprofile between antibody Sha31 and antibody SAF84. Furthermore, H-type BSE showed a fourth band of low molecular mass when antibody SAF84 was applied (Fig. 1, arrow). In addition of the absence of 12B2 reactivity as mentioned above, the analysed L-type showed a glycoprofile with a high proportion of monoglycosylated band (Sha31: 39% vs. 17% for C-type, Fig. 1).

The analyses of 39 bovines from the Belgian BSE archive did not yield any atypical case. As main evidence for discrimination, the glycoprofiles of all the samples exhibited a typical C-type glycoprofile with usual antibodies with >55% diglycosylated PrPres band. To further exemplify this, the glycoprofiles of the individual samples as probed with antibody 94B4 have been plotted in Fig. 2 and compared with the L-type and H-type results. In this figure, it is clear that the glycoprofiles of the samples did correspond to that of C-type, as characterized by a fraction of diglycosylated PrPres of 55% or more using 94B4 antibody; the L-type and H-type showed diglycosylated fractions below 50%. Therefore, all cases of age 7 years and older have the features of C-type BSE. Our previously reported case reinvestigated with the current improved tools lead to the conclusion that this case does belong to the C-type category.

Discussion

The results shed some light on the worldwide prevalence of atypical BSE. The absence of atypical cases can be linked to the limited sample size due to the dimension of Belgium, and is also likely to occur when comparing the variable prevalences found in other countries like France (8 L-types, 8 H-types), Poland (6 L-types, 1 H-type), Italy (3 L-types), The Netherlands (2 L-types, 1 H-type), Germany (1 L-type, 1 H-type), United Kingdom (2 H-types), Switzerland (1 H-type) and Catalunya, Spain (none). In all these countries, all cattle of 7 years and older have been tested post-mortem for the presence of BSE using sensitive screening methods. An extensive study already estimated the frequency of H-type and L-type BSE in France to 1.9 and 1.7 per million cattle more than eight years of age. This corresponds to 0.41 and 0.35 atypical cases per million of tested cattle. With almost three million cattle tested in Belgium since 1997, one could have expected to find roughly one or two H-type and one L-type (i.e. 1 atypical case every four or five years). It is also possible though difficult to prove that, atypical cases have been missed during the routine surveillance programme due to 1) an unusual location of the PrP^res deposition in the brain since at least L-types have a preferential distribution of PrP^res in the forebrain region and active surveillance methods are based on the brainstem; and 2) a susceptibility of critical epitopes of PrP^res in L- and/or H-type cases to proteinase treatment used for detection combined with the method used for initial screening (TeSeE ELISA of Bio-Rad). With respect to the latter point, screening tests have been developed using C-type cases which appear to be more resistant to digestion with proteinase K than H- and L-type cases. However, it must be mentioned that a significant
proportion of L- and H-type cases were detected using this type of capture ELISA, the most frequently test used up till 2007. This issue of suitability of screening tests needs nevertheless more attention in future studies when sufficient material is available e.g. from experimentally infected animals.

The age of tested cohort is a determining factor. For example, the cattle population in Poland is older than in other European countries and this country presents high atypical-BSE prevalence (especially of L-types). However this does not fully explain the prevalence of atypical BSE, as The Netherlands have a higher prevalence with an age structure of bovine population similar to that of Belgium. For now, the importance of the spontaneous aspect of (atypical) BSE remains an open question. The frequencies of atypical BSE cases are similar to those of the human sporadic Creutzfeldt-Jakob disease (sCJD). Indeed atypical cases were detected in BSE-exposed countries (France, Italy, Germany, The Netherlands, etc.) as well as in a low BSE-exposed country (Sweden). This reinforces the hypothesis of a sporadic origin of atypical BSE. These atypical BSE cases might have existed previously and by their very low frequency remained undetectable for veterinarians, until the introduction of the active surveillance programmes and improvements of diagnostic tools.

The origin of the current BSE epidemic could be linked to atypical BSE, especially in view of the properties of L-type. It is however also not excluded that the real source of the epidemic was derived from C-type case (which might also have a spontaneous origin), and thus that such cases have always existed sporadically. In such situation when continuing surveillance, C-type cases will be detected in older animals at a stable sporadic level. Potentially brain stem might not be the first site for PrPres development.

About the typing technique used in the present study, it is important to note that the apparent molecular mass differences can be useful but present as rather imprecise and impractical criteria for molecular typing of PrPres. The H-types can be discriminated with that measure if compared with C-types (Fig. 1a). However, given the precision of the Western blot technique, the differences in apparent molecular mass of PrPres for L-types are too small to be undoubtedly detected (0.3 and 0.8 kDa). A powerful discrimination tool remains the comparison of glycoprofiles, i.e. the proportions of the three forms of the PrP. L-types and C-types differ by their proportion of diglycosylated PrPres, which is respectively at/below and above 50% of total PrPres.

Conclusions

The emergence of atypical types of BSE is due to the active surveillance screening, a better awareness of prion strain variations, and more efficient diagnostic techniques. However considering molecular properties, PrPres in atypical cases is more susceptible to proteinase K treatment and the area in the brain where PrPres is deposited differs at least between C- and L-types. It is therefore essential to ascertain that the routine sampling and analytical techniques are adapted to these new types. As these new strains seem more virulent than classical types, at least in mice models, they represent one of the next challenges in the field of prions.
This analysis of the old bovines in the Belgian archive did not show any atypical BSE case in these cohorts. The study implied 39 bovines of at least 7 years of age. Even with the restricted size of Belgium, one could have expected a few atypical cases (as observed in The Netherlands). This difference can be random or linked to an unknown particularity of the Belgian samples. Anyway, the results help to estimate the worldwide prevalence of atypical BSE.

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