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Isolation from Cattle of a Prion Strain Distinct from That Causing Bovine Spongiform Encephalopathy

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To date, bovine spongiform encephalopathy (BSE) and its human counterpart, variant Creutzfeldt-Jakob disease, have been associated with a single prion strain. This strain is characterised by a unique and remarkably stable biochemical profile of abnormal protease-resistant prion protein (PrP^{res}) isolated from brains of affected animals or humans. However, alternate PrP^{res} signatures in cattle have recently been discovered through large-scale screening. To test whether these also represent separate prion strains, we inoculated French cattle isolates characterised by a PrP^{res} of higher apparent molecular mass—called H-type—into transgenic mice expressing bovine or ovine PrP. All mice developed neurological symptoms and succumbed to these isolates, showing that these represent a novel strain of infectious prions. Importantly, this agent exhibited strain-specific features clearly distinct from that of BSE agent inoculated to the same mice, which were retained on further passage. Moreover, it also differed from all sheep scrapie isolates passaged so far in ovine PrP-expressing mice. Our findings therefore raise the possibility that either various prion strains may exist in cattle, or that the BSE agent has undergone divergent evolution in some animals.

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Introduction

While transmissible spongiform encephalopathies (TSEs) in small ruminants and humans are believed to involve distinct prion strains [1,2], a single prion strain has been associated so far with bovine spongiform encephalopathy (BSE) and its human counterpart, variant Creutzfeldt-Jakob disease (vCJD) [3–6]. In particular, the abnormal, protease-resistant form of prion protein (PrP^{res}) that accumulates in the brains of infected individuals [7] shows a consistently unique electrophoretic profile in immunoblots [8]. However, the biochemical testing of the brains of slaughtered and fallen cattle, which was intensified since 2000 in European countries as a means to protect the consumers, has led to the discovery of positive samples that showed distinct PrP^{res} profiles. These atypical profiles have been sorted into two groups so far, provisionally termed H-type when the size of the protease resistant fragments is higher than for BSE, and bovine amyloidotic spongiform encephalopathy, or L-type, when it is lower [9,10]. These observations raise the possibility that as yet unrecognised prion strains may exist in cattle as in other species [11], and have potential implications in terms of public health. Unlike bovine amyloidotic spongiform encephalopathy isolates, which derive from animals with defined histopathological abnormalities [10], precise information corroborating a prion disease is lacking for H-type cases. It was therefore crucial to determine through experimental transmission whether such cases reflect some alteration in PrP metabolism, possibly in aging animals, or involve a truly infectious agent.

In this study, we report the transmission of a TSE-like disease by inoculation of French cattle isolates identified as

H-type variants to two lines of PrP transgenic mice. Furthermore, we provide compelling evidence that this agent has unique features compared to epizootic BSE and other related agents. We also establish that there is no link with ovine TSE isolates transmitted so far to these models.

Results

H-Type Isolates Are Transmissible to Mice

Two transgenic mouse lines were used as recipient for transmission experiments. The tg540 line is a newly established line that expresses bovine PrP (Protocol S1), resulting in an enhanced susceptibility to BSE agent compared to conventional mice [6,12]. The tg338 line, expressing the VRQ (Val¹³⁶Arg¹⁵⁴Gln¹⁷¹) allele of ovine PrP, has allowed an efficient transmission of natural scrapie isolates from sheep and goat [13,14]. The rationale for including tg338 mice in this study was the possibility that characterisation of a prion

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Abbreviations: BSE, bovine spongiform encephalopathy; PrP^{res}, protease-resistant prion protein; TgBov, bovine tg540; TgOv, ovine tg338; TSE, transmissible spongiform encephalopathies; vCJD, variant Creutzfeldt-Jakob disease

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Synopsis

Prions are unconventional agents of proteic nature that are formed of abnormal conformations of the host-encoded prion protein (PrP). They cause fatal neurodegenerative diseases in both animals and humans, and can be transmitted between species as exemplified in humans by the emergence of variant Creutzfeldt-Jakob disease following the epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom. Since diagnosis of prion infection is only possible once the central nervous system has been invaded, brains of slaughtered or fallen cattle are routinely screened in Europe to protect the consumers from BSE. This has unexpectedly led to the discovery of unprecedented PrP conformations that were distinct from the single one associated so far with BSE or BSE-related diseases. To precisely determine their etiology, the authors have studied the transmissibility of these new conformations, termed H-type, to transgenic mice expressing either bovine or ovine PrP. They show that these cases are highly pathogenic for these mice. The authors also demonstrate that they are not directly related to the agent involved in the BSE epidemic, supporting the view for isolation of a new prion strain from cattle, whose prevalence and associated zoonotic risk should be carefully monitored in the future.

accidentally passed from small ruminants to cattle might be facilitated on such mice, by comparison with the ovine isolates transmitted so far. Tg540 (tgBov) and tg338 (tgOv) mice overexpress PrP in the brain at similar levels (~8- to 10-fold). Both lines have a normal lifespan, the same as PrP^{0/0} mice on which the transgenes were introduced. H-type isolates representative of a series of seven samples identified in France were inoculated intracerebrally to tgBov and tgOv mice (Table 1). Typical BSE agents from cattle and from other species was inoculated to the same mice for the sake of comparison. Remarkably, all H-type isolates induced a neurological disease on primary transmission, with a 100%

attack rate in both mouse lines. The mean survival times observed with cases no. 1 and no. 2 in tgBov mice, ~400 d, appeared to be prolonged compared to those for cattle, sheep, goat BSE, and human vCJD inocula, which ranged from ~250 to 380 d. Such a discrepancy could reflect a lesser infectivity of H-type samples, consistent with their comparatively lower PrP^{res} content [9]. Moreover, the survival time was reduced by ~100 d on subpassage, approaching that for BSE from cattle or other sources on secondary passage, or on primary passage for inocula of presumably higher titre (i.e., producing no substantial reduction of survival time on subpassage: BSE no. 3 and ARQ [Ala¹³⁶Arg¹⁵⁴Gln¹⁷¹] no. 1). Upon transmission to tgOv mice, the mean incubation period produced by the four H-type cases was strikingly homogeneous (586–612 d), consistent with a potentially unique agent (Table 1). This was comparable to or even shorter than the incubation periods of epizootic BSE or related inocula on the same mice (560–792 d). As illustrated in Figure 1, the relative incubation periods observed on tgOv and tgBov mice appeared to differ significantly among the H-type and BSE-type agents. In addition, the reduction in incubation period observed upon secondary transmission of H-type (case no. 2) on tgOv mice was significantly less dramatic than that observed for vCJD and sheep BSE inocula (Table 1). Overall, these suggested that H-type and BSE might be different TSE agents.

H-Type PrP^{res} Profile Is Preserved in Transgenic Mice

The brains of diseased mice were analysed by immunoblotting for the accumulation of abnormal PrP. PrP^{res} was readily detected in all mice tested since the first passage, consistent with the efficient transmission observed in both lines (10/10 and 33/33 positive brains for tgBov and tgOv mice, respectively). The PrP^{res} molecular profile was fairly uniform

Table 1. Transmission of Bovine Molecular Variant Cases (H-Type) to Transgenic Mice Expressing Bovine or Ovine PrP

Isolate	Case Number	Passage	Mean survival time, d ± SEM (n/n ₀) ^a	
			tgBov Mice	tgOv Mice
H-type	1	First	414 ± 10 (5/5)	612 ± 26 (10/10)
		Second	317 ± 6 (8/8)	ND
	2	First	401 ± 9 (5/5)	595 ± 18 (8/8)
		Second	296 ± 3 (9/9)	319 ± 10 (6/6)
	3	First	ND	607 ± 12 (6/6)
5	First	ND	586 ± 15 (9/9)	
BSE	1	First	377 ± 22 (6/6)	ND
	3	First	298 ± 7 (9/9)	704 ± 36 (7/7)
		Second	283 ± 10 (5/5)	NA
Sheep BSE^b	ARQ 1	First	278 ± 2 (6/6)	560 ± 60 (5/5)
		Second	263 ± 6 (6/6)	178 ± 2 (4/4)
	ARQ 3	First	339 ± 25 (5/5)	ND
Goat BSE^c	ARR 1	First	340 ± 8 (7/7)	ND
	CH636	First	253 ± 9 (6/6)	590 ± 43 (4/4)
Variant CJD	NHBY0/0003	First	343 ± 8 (5/5)	792 ± 22 (6/6)
		Second	293 ± 11 (6/6)	195 ± 9 (6/6)
Control	Sheep brain	First	793 ± 26 (0/9)	835 ± 15 (0/6)

NA, not available; ND, not done.

^aIntracerebral inoculation with 2 mg brain tissue equivalent; n/n₀: diseased/inoculated.

^bExperimental cases.

^cField case.

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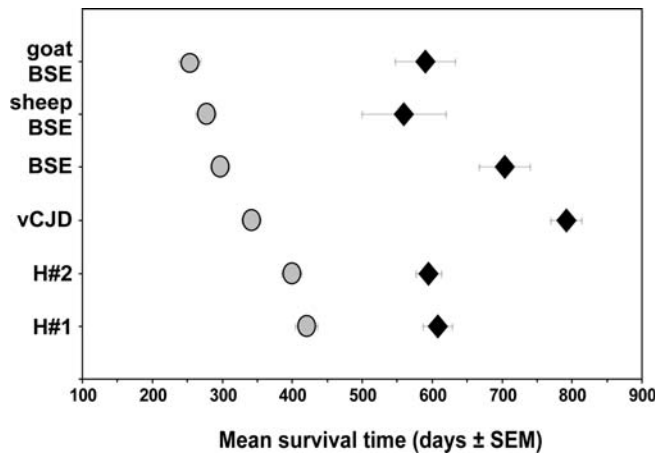


Figure 1. Survival Time in Transgenic Mice Infected with H-Type and BSE-Type Agents

Mean survival times (days \pm SEM) upon primary transmission are shown for tgBov (grey circles) and tgOv (black diamonds) mice inoculated with H-type cases, BSE, and related isolates (see Table 1). The intervals between the incubation times on each line are significantly different for H-type and BSE agents ($p < 0.0002$, Fisher test). DOI: 10.1371/journal.ppat.0020112.g001

among the isolates. Remarkably, like the BSE agent for which the typical signature was conserved whatever the donor species (≥ 3 brains analysed per combination), the H-type agent essentially retained its biochemical phenotype upon serial transmission to tgBov as well as to tgOv mice expressing a heterologous PrP^C (Figure 2 and below). Compared to BSE PrP^{res}, it was characterised by a significantly higher apparent molecular mass (difference measured for unglycosylated species: 0.9 ± 0.05 kDa and 0.7 ± 0.06 kDa in tgBov and tgOv mice, respectively) and the relative proportions of glycoforms were essentially similar. A further difference was the lack of detectable PrP^{res} in the spleen of H-type diseased tgOv mice (three to five spleens tested per isolate), while this accumulated at substantial levels after BSE or vCJD infection (Figure 2B).

H-Type and Epizootic BSE Agents Exhibit Distinct Neuropathological Features

We next examined the PrP^{res} targeting and the vacuolation in the brain, which are known to exhibit a strain-dependent variation [6,15,16]. This was performed on tgBov mice since they express a PrP^C homologous to that of the donors, including the number of octarepeats [17], thus providing a relevant context for comparing H-type and epizootic BSE isolates. H-type isolates showed a similar distribution of PrP^{res} deposits on both primary and

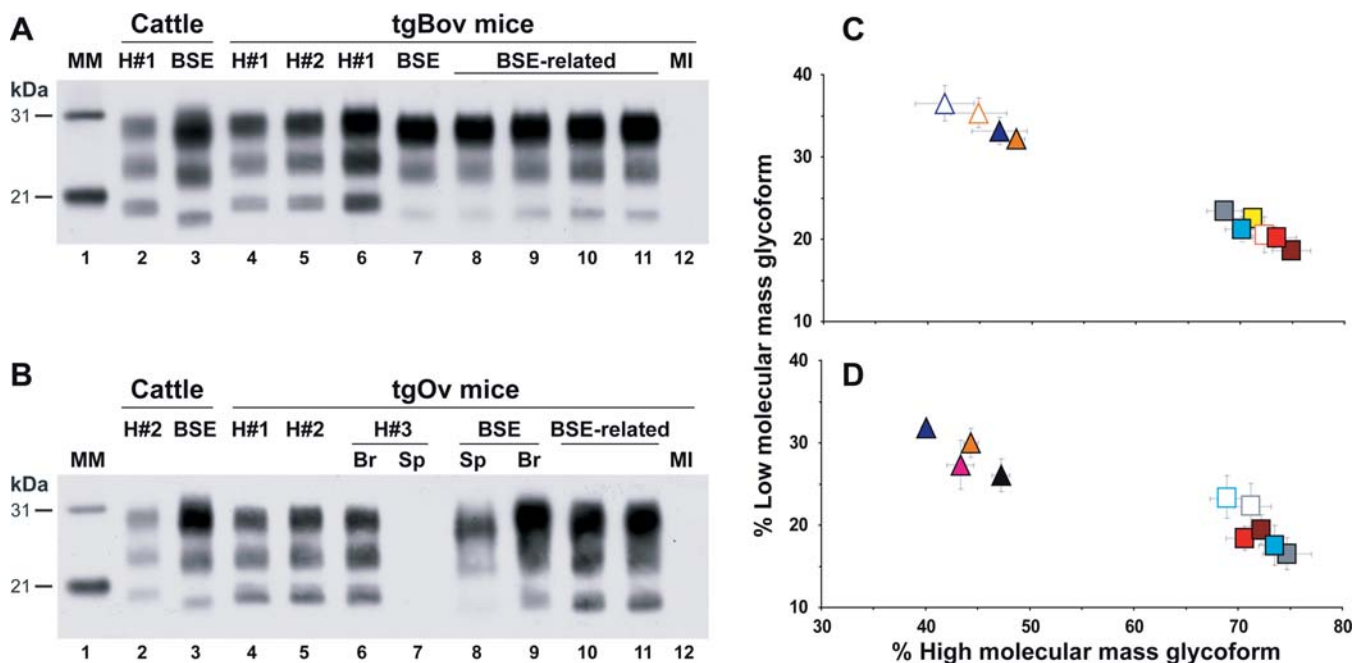


Figure 2. Western Blot Analysis of PrP^{res} in the Brains of Transgenic Mice Infected with H-Type or BSE-Type Agents

(A) Primary or secondary (lane 6) transmission to tgBov mice. BSE-type inocula include cattle BSE no. 3 (lanes 3 and 7), sheep BSE ARQ no. 1 (lanes 8 and 9), goat BSE (lane 10), and vCJD (lane 11). The PrP^{res} profiles of both H-type and BSE agents in cattle (lanes 2 and 3) are essentially similar to those in tgBov mice (lanes 4–11). Brain tissue equivalent loaded: 2.5 mg in lane 2; 0.15 mg in lane 3; 0.5 mg in lanes 4–12. MI, mock-infected brain; MM, molecular markers.

(B) Primary transmission to tgOv mice. H-type agent shows a distinct PrP^{res} pattern in the brain (Br) compared to BSE agents (lane 9, BSE no. 3; lane 10, goat BSE; lane 11, vCJD). Note the lack of PrP^{res} signal in the spleen (Sp) of H-type-infected mice (lane 7), unlike that in BSE-infected mice (lane 8). Brain or spleen tissue equivalent loaded: 3 mg in lane 2; 0.15 mg in lane 3; 0.5 mg in lanes 4–6; 2 mg in lanes 7–12.

(C and D) Ratio of high- and low-molecular-mass PrP^{res} glycoforms in the brains of tgBov (C) and tgOv (D) mice following challenge with H-type or BSE agents (data plotted as means \pm SEM). H-type isolates are represented as triangles (no. 1, blue; no. 2, orange; no. 3, pink; and no. 5, black) and BSE agents as squares (BSE no. 3, red; sheep BSE ARQ no. 1, grey; sheep BSE ARR no. 1, yellow; goat BSE, brown; and vCJD, light blue). Secondary transmissions are represented by unfilled symbols of the same colour. Note the strikingly distinct glycoform ratio between H-type and BSE groups in both mouse lines, as reported in cattle [9]. DOI: 10.1371/journal.ppat.0020112.g002

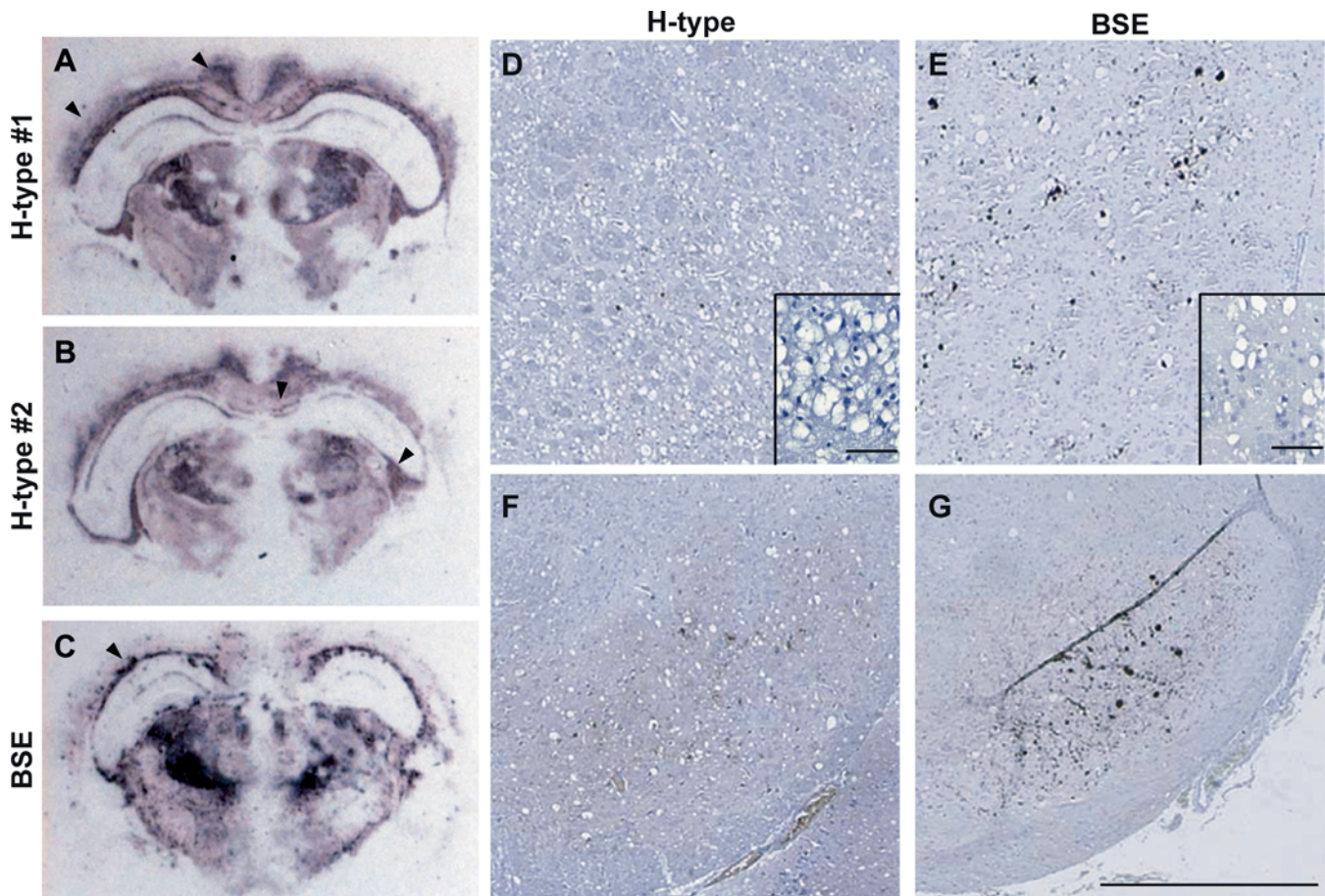


Figure 3. Regional Distribution of PrP^{res} and Vacuolar Changes in the Brains of Bovine Transgenic Mice Infected with H-Type or BSE Agents

Histoblots of representative coronal sections of tgBov mouse brains at the levels of the hippocampus are shown. The distribution of PrP^{res} deposits was similar among H-type isolates (A) (B), and different from that of cattle BSE (C) in several areas indicated by arrowheads, such as the cortex, the corpus callosum and dorsal commissure, alveus, fimbria, and stratum oriens of the hippocampus. Note that intensity of PrP deposition markedly differed between H-type and BSE agents. Illustration of how this appears by immunohistochemistry in the striatum (D) (E) and substantia nigra (F) (G), H-type-infected mice being less intensively labelled than those infected with BSE agent. By contrast, spongiosis was much more severe in H-type-infected brains. Bars: 30 μ m; insert: 7 μ m.

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secondary transmission, as assessed by histoblotting on brain coronal sections (Figure 3A–3B). The staining did not differ from that seen with cattle BSE in several regions, such as the striatum and several nuclei of the thalamus, including the geniculate, ventral postero-lateral and -medial as well as the brain stem (Figure 3A–3C and data not shown). However, other areas such as the cerebral cortex, the corpus callosum including the cingulum, the dorsal commissure, the alveus, and fimbria of the hippocampus were predominantly stained with H-type, whereas BSE PrP^{res} was rather confined in the stratum oriens of the hippocampus (Figure 3A–3C). Moreover, the overall intensity and aspect of PrP deposition markedly differed between the two types of agents. While immunochemistry revealed various types of PrP deposits in both cases, thin diffuse PrP deposits were predominant in H-type-infected brains, whereas the most frequent type was granular in BSE-infected mouse brains. In several areas, including the striatum and the substantia nigra (Figure 3D–3G), there was a striking lack of correlation between the intensity of PrP deposition and the severity of the vacuolation. Overall, the vacuolation was much more intense

in the case of H-type variant (Figures 3 and 4): areas such as the septum, hypothalamus, hippocampus, and cortex showed severe spongiosis, accompanied by a pronounced reactive glial astrocytosis based on GFAP staining (not shown), while BSE-induced vacuolation was moderate in the same areas.

H-Type Agent Is Distinct from the Ovine TSE Isolates Transmitted so Far to tgOv Mice

We finally examined whether H-type isolates may have an ovine TSE origin. The majority of typical and atypical sheep scrapie isolates we have studied so far transmits before a year to tgOv mice ([13,14] and our unpublished data). Only a group of sheep scrapie isolates from Italy (SSit) was found to infect tgOv mice after a prolonged survival time within the range of H-type cases. Indeed three of them, SSit cases no. 5, no. 7, and no. 8, induced a typical neurodegenerative disease with a mean survival time of 698 ± 20 d (5/5 animals affected), 659 ± 31 d (7/7), and 569 ± 37 d (4/4), respectively. Case no. 5 incubation time was still longer than H-type case no. 2 on subpassage (417 ± 20 d, 6/6 animals affected). The PrP^{res} molecular profile observed in the brain of SSit-diseased mice

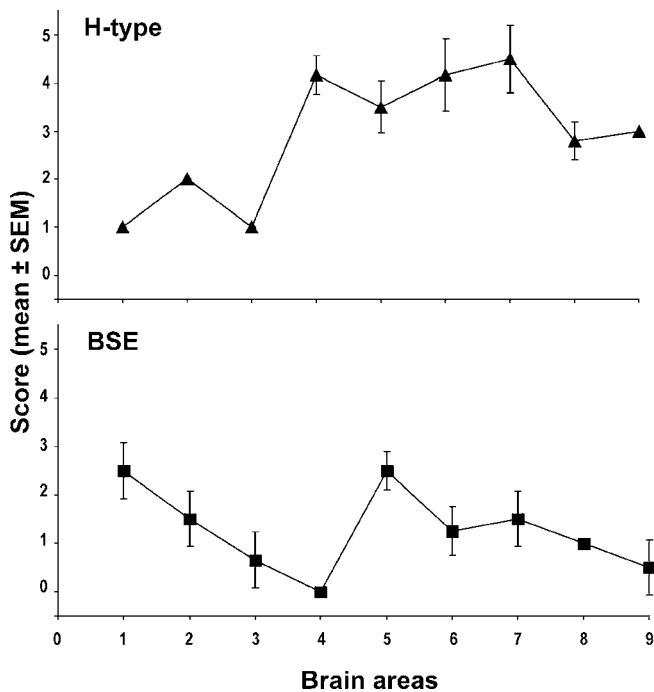


Figure 4. Lesion Profiles in tgBov Mice Infected with H-Type or BSE Agents

Mean scores (\pm SEM) reflecting the intensity of vacuolation are shown for H-type (no. 1 and no. 3, triangles) and BSE (no. 1 and no. 3, squares). The nine grey-matter areas used to construct the profile are as follows: dorsal medulla (1); cerebellar cortex (2); superior colliculus (3); hypothalamus (4); medial thalamus (5); hippocampus (6); septum (7); medial cerebral cortex at the level of the thalamus (8); and medial cerebral cortex at the level of the septum (9). DOI: 10.1371/journal.ppat.0020112.g004

(more than four brains analysed per isolate) was characterised by a significantly higher apparent molecular mass (difference of 0.7 ± 0.2 kDa) and a slightly higher proportion of diglycosylated PrP^{res} as compared with H-type-derived PrP^{res} (Figure 5A–5C). These features were conserved on secondary transmission (Figure 5A–5C). Another difference was again the pronounced accumulation of SSit-associated PrP^{res} in the spleen, while this was still impaired in H-type-infected mice, even after two passages (three to five spleens tested per combination; Figures 2B and 5A).

We then compared by histoblot distribution and nature of PrP^{res} deposits within the brains of tgOv mice infected with a second passage of H-type (no. 2) and SSit (no. 5) cases. Both markedly differed between the two agents as illustrated in Figure 5. Indeed, large plaques of SSit-associated PrP^{res} were present in the pretectal nuclei and in related structures of the limbic system such the fornix, the alveus, fimbria, and subiculum of the hippocampus. H-type-associated PrP^{res} was detected instead in the corpus callosum, cortex, and ventromedial thalamic nuclei (Figure 5D and unpublished data). The deposits seem rather thin or granular. SSit case no. 8, which gave the shortest incubation period in tgOv mice, was also inoculated by intracerebral route to tgBov mice. Of note, no disease has been observed yet in mice monitored up to 600 d after infection. In conclusion, these data suggest that the H-type agent is unrelated to the ovine TSE isolates transmitted so far to our transgenic lines.

Discussion

In this study we show that cattle brain samples positive for abnormal PrP with a distinct molecular pattern, called H-type, consistently produces a fatal, TSE-like disease upon inoculation to both bovine and ovine PrP transgenic mice. These results, corroborating the recent transmission to wild-type mice [18], formally establish that such cases involve an authentic TSE infectious agent. Importantly, we provide detailed evidence that this newly recognised agent differs from epizootic BSE agent derived from cattle or other species.

Both molecular and biological criteria support the conclusion that H-type and BSE agents are distinct prion strains. First, the incubation periods upon transmission to mice expressing either bovine or ovine PrP produced different patterns. Thus, while primary transmission to tgOv mice led to longer survival times for both agents, the increase relative to tgBov mice was significantly less for H-type than for BSE-type agents (Figure 1). Second, the molecular profiles of the PrP^{res} fragments detected in the brain of diseased mice were clearly distinguishable in either line. Strikingly, differences observed in terms of fragment size and glycoform ratio were essentially the same as in cattle brain. Third, unlike that for BSE agents, no PrP^{res} signal could be seen in the spleen of H-type-infected tgOv mice, indicative of a stronger neurotropism at least in this host. Fourth, histopathological examination of tgBov mice revealed a contrasting picture. Typically, severe spongiosis and diffuse PrP deposition were present in several areas of H-type-infected brains, while the same areas of BSE-infected brains showed limited spongiosis together with marked PrP deposition. Such discrepancies are unlikely to result from unequal survival times since they were also observed on secondary passage, where the two agents had comparable incubation duration (unpublished data).

The isolation from cattle of a prion strain distinct from the one implicated in the BSE epidemics raises several concerns. One is whether H-type isolates might result from an exposure to prions of small ruminants via alimentary or environmental sources, since cattle have been shown to be susceptible to experimental infection by sheep scrapie agent [19]. In this regards, the better compatibility between ovine PrP sequence and H-type as compared to BSE was intriguing (Figure 1). However, our investigations do not support this. Among the five groups of natural isolates we have identified so far in tgOv mice ([13,14] and our unpublished data), only one group, made up mostly of SSit isolates, proposed to be of iatrogenic origin [20], showed an incubation time as prolonged as for H-type cases. However, the PrP^{res} molecular profile, nature of deposits, and distribution within the brain as well as the differential accumulation in the spleen strongly distinguish H-type and SSit isolates. In addition, the latter failed so far to transmit to tgBov mice.

H-type and BSE agents might be related despite their distinguishable phenotypes. The isolation of an additional strain upon exposure of transgenic or wild-type mice to the epizootic BSE agent has been reported [21], thus questioning its strain homogeneity. Also, molecular typing studies have revealed the presence of a minor, non-BSE-type PrP^{res} component in BSE- and vCJD-infected brains [22]. Hence, H-type isolates could arise from the preferential amplification in certain individuals of a subcomponent present in BSE

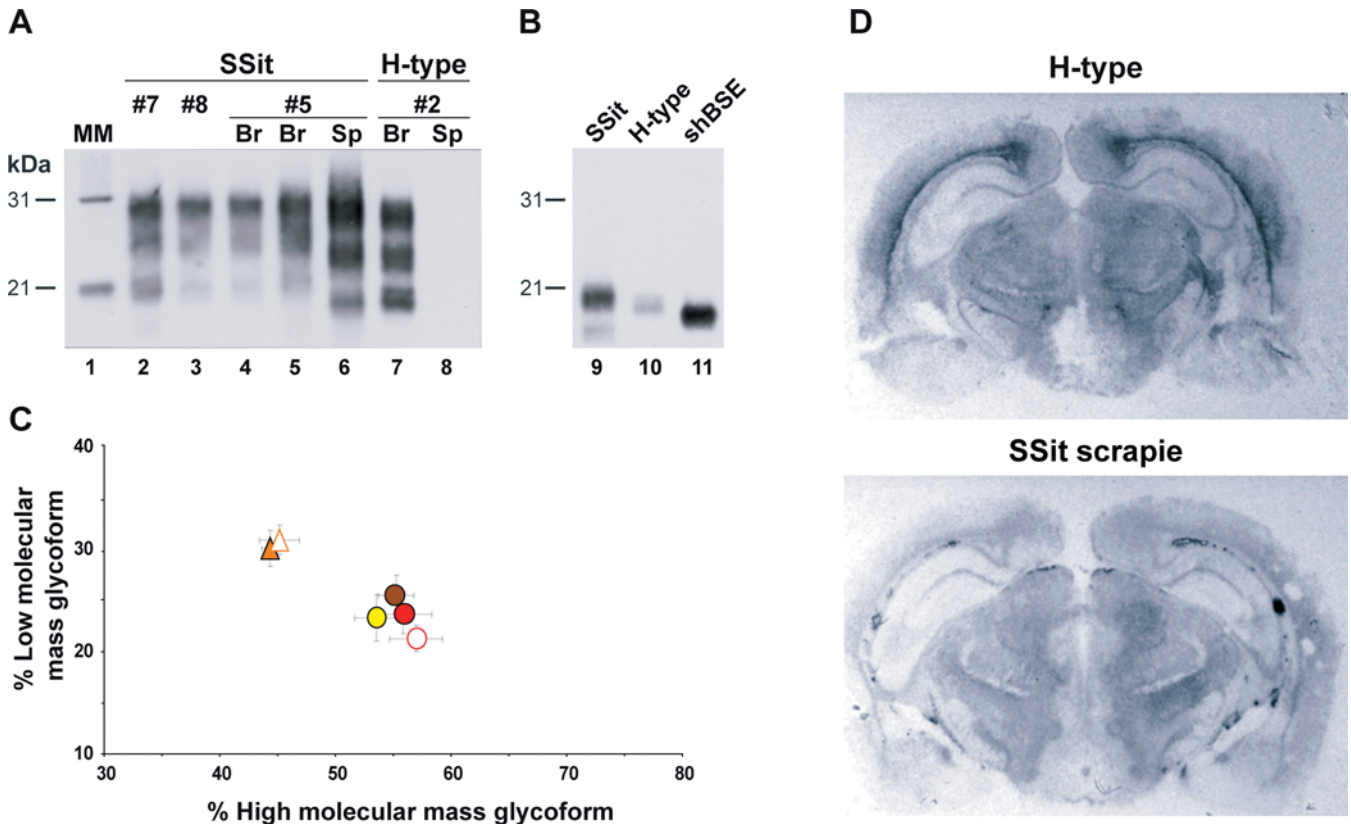


Figure 5. Comparison of H-Type and SSit Isolate Features upon Transmission to tgOv Mice

(A and B) Western blot analysis of PrP^{res} in the brains and spleens of tgOv mice infected with H-type or SSit isolates at first (lanes 2–4) and second passage (lanes 5–11). H-type PrP^{res} shows a distinct pattern in the brain (Br) compared to SSit. The apparent molecular mass of SSit PrP^{res} is higher than that of H-type or sheep BSE (shBSE), as shown after PNGase treatment (B). Note also that PrP^{res} is detected in the spleen (Sp) of SSit- but not of H-type-infected mice. Tissue equivalent loaded: 1.5 mg in lanes 2–4; 0.04 mg in lane 5; 0.5 mg in lanes 6–7; 2 mg in lane 8; 0.01 mg in lane 9; 0.1 mg in lanes 10–11. MM, molecular markers.

(C) Ratio of high- and low-molecular-mass PrP^{res} glycoforms in the brain of tgOv mice infected with H-type or SSit isolates (data plotted as mean \pm SEM). One H-type isolate (no. 2) is represented as orange triangle. SSit isolates are represented as circles (SSit no. 5, red; no. 7, brown; no. 8, yellow). Secondary transmissions are represented by unfilled symbols of the same colour. Note the stably distinct glycoform ratios between H-type and SSit agents upon serial passage.

(D) Regional distribution of PrP^{res} in the brain of tgOv mice infected with H-type or SSit isolates. Histoblots of representative coronal sections of tgOv mouse brains at the levels of the hippocampus are shown. The distribution of H-type-associated PrP^{res} deposits was different from that of SSit in regions such as the alveus of the hippocampus, the corpus callosum, the pretectal nuclei, the cortex, and the ventromedial thalamus. Note that the size of PrP^{res} deposits markedly differed between the two types of isolates.

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infectious sources. Comparing H-type and BSE-derived variant prions identified in mice might be informative in that respect.

Alternatively, such unusual cases could reflect the existence of a natural, sporadic disease in cattle. Although it is unclear yet if such infections may lead to a clinical disease in the natural host, they seem to occur at a low frequency, which is reminiscent of the situation known for sporadic CJD in humans [23,24]. Of note, the disparities between intensity of PrP deposition and severity of vacuolation in the brains of H-type-inoculated tgBov mice have also been observed with sporadic CJD both in human or mouse infected brains [21,25]. These data, however, need to be consolidated through further investigations, including epidemiological analysis. Indeed, an implication of this latter scenario is that such bovine “atypical” cases could occur in countries free of BSE exposure. The acquisition of novel properties by an endogenous, sporadic cattle TSE agent, as occasioned on passage through an intermediary host or a physicochemical treatment

such as that applied to carcass-derived products, has been invoked as one possible origin for the emergence of BSE epidemics [7]. With the isolation of such agents, we can now address this issue experimentally.

In conclusion, our findings support the view that at least two and potentially three [10] distinct prion strains may be present in cattle. The current uncertainties regarding the origin, prevalence, and potential risk for humans of a strain of TSE agent unrecognised until recently should support continued efforts to characterise it *in vivo* and uphold the surveillance exerted on cattle.

Materials and Methods

Isolates. The H-type [9], goat BSE (CH636 case [26]), and experimental sheep BSE samples [27] were provided by the French TSE Reference Laboratory (Agence Française de Sécurité Sanitaire des Aliments [AFSSA], Lyon, France). The samples from French BSE cases and from experimental sheep BSE (ARR [Ala¹³⁶Arg¹⁵⁴Arg¹⁷¹] genotype [28]) were provided by the Institut National de la Recherche Agronomique (INRA; Toulouse, France) and the Institute for Animal

Health (IAH) Neuropathogenesis Unit (Edinburgh, United Kingdom), respectively. The vCJD isolate was a World Health Organization (WHO) reference sample from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, United Kingdom). SSit isolates were provided by the Istituto Superiore di Sanita (ISS; Rome, Italy).

Transgenic mice and transmission assays. The tg540 line expresses the bovine PrP allele with 6 octarepeats under the control of the cytomegalovirus (CMV) promoter on a FVB mouse line with PrP⁰⁰ background (Protocol S1). The tg338 line expresses the V₁₃₆R₁₅₄Q₁₇₁ allele of ovine PrP at a homozygous state, on a mouse PrP⁰⁰ background [29]. The transgene construct (tg3) consists in a bacterial artificial chromosome (BAC) insert of 125 kb of sheep DNA [13]. All experiments were performed according to national guidelines. Each inoculum was prepared extemporaneously in a class II microbiological cabinet using disposable equipment. Individually identified 6- to 10-wk-old mice were inoculated intracerebrally with 20 µl of a 10% (wt/vol) brain homogenate in 5% glucose. Mice were monitored daily once ill and killed in extremis.

Analysis of PrP^{res} molecular pattern. All procedures regarding purification and detection of PrP^{res} from brains and spleens of infected mice were as described [14]. ICSM18 [30] or Sha31 [31] anti-PrP antibodies were used. Enzymatic deglycosylation was performed on denatured PrP^{res} with 1,000 U of recombinant PNGase (New England Biolabs, Beverly, Massachusetts, United States) for 2 h at 37 °C in 1% Nonidet P40 and the proprietary buffer as described [30]. Determination of glycoform ratio and apparent molecular mass was performed with the GeneTools software after acquisition of chemiluminescent signals with a GeneGnome digital imager (SynGene, Frederick, Maryland, United States).

Histopathology. For histoblot analysis [32], brains were rapidly removed from killed mice and frozen on dry ice. Thick 10-µm cryostat sections were cut, transferred onto Superfrost slides, and kept at -20 °C until use. The procedure was performed as described [14] using the 12F10 anti-PrP antibody [33]. All immunohistochemistry procedures regarding tissue processing have been described previously [34]. Samples were fixed in neutral-buffered 10% formalin (4% formaldehyde) before paraffin embedding. After deparaffinisation, 6-µm-thick tissue sections were stained with haematoxylin/eosin. Vacuolation profiles were established, following the standard method

described by Fraser and Dickinson [15], by using two to three brains per isolate.

Supporting Information

Protocol S1. Description of the Bovine PrP Transgenic Mice (tg540 Line)

Found at DOI: 10.1371/journal.ppat.0020112.sd001 (92 KB DOC).

Accession Numbers

The GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) accession numbers for the genes and gene products discussed in this paper are bovine PrP (NM181015) and sheep PrP (M31313).

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