Background: The cause of the bovine spongiform encephalopathy (BSE) epidemic in the United Kingdom (UK) was the inclusion of contaminated meat and bone meal in the protein rations fed to cattle. Those rations were not restricted to cattle but were also fed to other livestock including farmed and free living deer. Although there are no reported cases to date of natural BSE in European deer, BSE has been shown to be naturally or experimentally transmissible to a wide range of different ungulate species. Moreover, several species of North America's cervids are highly susceptible to chronic wasting disease (CWD), a transmissible spongiform encephalopathy (TSE) that has become endemic. Should BSE infection have been introduced into the UK deer population, the CWD precedent suggested a danger for spread and maintenance of the disease in both free living and captive UK deer populations. This study examined the immunohistochemical and biochemical characteristics of BSE experimentally-infected European red deer (*Cervus elpahus elaphus*).

Annex A (*BMC Veterinary Research* 2008, **4**:17) and annex B *(BMC Veterinary Research* 2009, **5**:26) on page 4 contain the technical details of this report up to July 2009 and were published in BMC veterinary Research a peer reviewed electronic open access journal. Pages 2-3 detail additional work undertaken between July 2009 and March 2010.

Methodology

18 red deer were orally dosed with 25g of cattle BSE homogenate and 6 deer were intra-cerebrally (i.c.) inoculated with 0.5 ml of a 10% homogenate of a brain pool of five BSE-positive bovine brains. Negative control animals underwent identical procedures to the i.c and orally dosed deer, respectively, with sterile normal saline instead of brain homogenate. A sensitive IHC method employing monoclonal antibodies able to differentiate between BSE in cattle and CWD in deer was optimised prior to the first deer culls at 6 and 12 months post inoculation (mpi). The full range of CNS, LRS, PNS and other tissues taken at PM were then examined by IHC and brainstems by Western blot.

Results

No pre-clinically diseased orally infected animals were identified at the 6 months and 12 months post infection cull points. Intra-cerebrally infected animals were maintained until all 6 developed clinical disease. Only one out of six orally dosed deer developed clinical disease before termination of the project.

We confirmed that U.K red deer are susceptible to both oral and intra-cerebral inoculation with the cattle BSE agent. Six clinically positive (from 26-42mpi) i.c inoculated and one (56mpi) orally dosed deer that tested positive for TSE by IHC and WB using several primary antibodies demonstrated widespread accumulation of disease specific PrP in CNS, PNS and ENS but none in LRS. All showed several brain sites positive for disease specific PrP and presented IHC and WB phenotypes with similarities to BSE in sheep, goats and cattle but unlike those seen in CWD in elk or scrapie in sheep. The vacuolar pathology and distribution of PrP^d BSE in red deer resembled that of CWD in most major respects however we have shown that BSE can be clearly differentiated from CWD by existing immunohistochemical and biochemical methods that are in routine use.

Further work undertaken August 2009 – March 2010.

Genetic analysis - Wilfred Goldmann; Roslin NPD.

Negative controls and the remaining 5 orally dosed deer culled at 72mpi tested negative by IHC and Western blot however analysis of the PrP ORF of these deer *(kindly carried out by Wilfred Goldmann of the Roslin NPD*) identified a Q to E polymorphism at codon 226 that may influence the efficiency of oral transmission (not published).
In the experimental BSE challenge of red deer six out of six deer succumbed to BSE when challenged by intracerebral routes but only one of six deer challenged by the oral route succumbed to infection. Deer killed at 190 days or 365 days post oral challenge showed no evidence of abnormal PrP accumulation when tested by immunocytochemistry. The PrP gene of red deer includes a Q to E polymorphism at codon 226. The table shows the distribution of these codon 226 polymorphisms within experimental challenge groups.

Deer ID	R No.	Challenge	Death	codon 226	IHC	**Deer ID**	R No.	Challenge	Death	codon 226	IHC
020	1791		int. death 210	QE	N	323	2160	oral	seq. kill 365	QE	N
322	1820		seq. kill 190	QQ	N	337	2161	oral	seq. kill 365	EE	N
319	2137		seq. kill 365	QE	N	314	2162	oral	seq. kill 365	QQ	N
334	2138		seq. kill 365	EE	N	318	2163	oral	seq. kill 365	QE	N
022	4173	ic saline	seq. kill 1017	EE	N	310	2164	oral	seq. kill 365	QE	N
029	4644	ic saline	seq. kill 1290	QE	N	028	2165	oral	seq. kill 365	QQ	N
032	5863		cull 2320	QQ	N						
034	5861		cull 2320	QE	N	312	3991	ic	TSE 794	EE	P
039	5864		cull 2320	EE	N	332	4140	ic	TSE 929	QE	P
014	5862		cull 2320	EE	N	329	4174	ic	TSE 996	QE	P
						331	4175	ic	TSE 996	QE	P
327	1813	oral	seq. kill 190	QE	N	025	4279	ic	TSE 1059	QQ	P
309	1821	oral	seq. kill 190	EE	N	315	4643	ic	TSE 1289	QE	P
031	1822	oral	seq. kill 190	QQ	N						
320	1823	oral	seq. kill 190	QE	N	009	4987	oral	TSE 1727	QQ	P
324	1824	oral	seq. kill 190	QQ	N	015	5866	oral	cull 2320	QE	N
326	1825	oral	seq. kill 190	QQ	N	033	5867	oral	cull 2320	EE	N
						016	5868	oral	cull 2320	EE	N
						023	5869	oral	cull 2320	QE	N
						037	5870	oral	cull 2320	EE	N

Death: numbers indicate age (oral controls) or time post-inoculation in days

Within the intracerebral challenge experiment QQ (n=1), QE (n=4) and EE (n=1) deer all succumbed to clinical BSE infection which was confirmed by histology and immunocytochemistry. These data show that the codon 226 polymorphism does not provide absolute resistance to infection. Although the first deer to succumb to infection was the only EE genotype it had to be killed early because of intercurrent health problems and had substantially less abnormal PrP in brain than the remainder of the group. Overall, the small number of deer within each genotype group does not permit any meaningful analysis of attack rates or incubation periods.

Of the oral challenged deer only one QQ deer succumbed to infection at 58 months post challenge while QE (n=2) and EE (n=3) deer did not show clinical signs of disease when the experiment was terminated at 72 months post challenge. The five deer that lived to the end of the experiment did not show histological or immunocytochemical evidence of disease.

These data are summarised in the table. There is insufficient information on which to draw unambiguous conclusions regarding the impact of the 226 polymorphism on susceptibility of red deer to BSE challenge, but the possibility that this codon may influence the efficiency of oral transmission cannot be dismissed. It would be prudent to take the frequency distribution of this polymorphism into consideration when performing critical analysis of deer surveys and possible interpretations of the incidence of deer TSEs within Europe.

Transgenic mouse bioassay - Marion Simmons/John Spiropoulos; VLA Weybridge.

2 BSE infected red deer plus the BSE source that was used to inoculate the deer were inoculated into a panel of 20 Tg(CervinePrP)1536$^{+/-}$ mice – not published. The deer passaged BSE bioassays have been completed and the bovine BSE source passage is ongoing. For deer 1 the attack rate was 18/20=90%. The incubation period varied from 202-274 days post inoculation (dpi) or 240$\pm$22 mean$\pm$standard deviation (M$\pm$SD). For deer 2 the attack rate was 19/20=95%. Incubation periods varied from 207-298 (dpi) or 254$\pm$28 (M$\pm$SD). A T-test analysis between the 2 groups showed no significance (p=0.096). The panel that was inoculated with the bovine BSE has surviving mice (n=12) 590 dpi. Seven of the mice died due to intercurrent deaths. Data analysis will be completed when the bioassays finish. It is however interesting that the deer succumbed to bovine BSE while the cervid mice show such prolonged incubation period.

Acknowledgements

Mark P Dagleish; Moredun Research Institute – Deer management / Post Mortems.
Wilfred Goldmann; Roslin NPD – Genetic analysis.
Marion Simmons/John Spiropoulos; VLA Weybridge – Transgenic mouse bioassay.

Publications

Annex **A)**

Experimental transmission of bovine spongiform encephalopathy to European red deer (*Cervus elaphus elaphus*)

Mark P Dagleish*[1], Stuart Martin[2], Philip Steele[1], Jeanie Finlayson[1], Sílvia Sisó[2], Scott Hamilton[1], Francesca Chianini[1], Hugh W Reid[1], Lorenzo González[2] and Martin Jeffrey[2]

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Abstract

Background: Bovine spongiform encephalopathy (BSE), a member of the transmissible spongiform encephalopathies (TSE), primarily affects cattle. Transmission is via concentrate feed rations contaminated with infected meat and bone meal (MBM). In addition to cattle, other food animal species are susceptible to BSE and also pose a potential threat to human health as consumption of infected meat products is the cause of variant Creutzfeldt-Jakob disease in humans, which is invariably fatal. In the UK, farmed and free ranging deer were almost certainly exposed to BSE infected MBM in proprietary feeds prior to legislation banning its inclusion. Therefore, although BSE has never been diagnosed in any deer species, a possible risk to human health remains via ingestion of cervine products. Chronic wasting disease (CWD), also a TSE, naturally infects several cervid species in North America and is spreading rapidly in both captive and free-ranging populations.

Results: Here we show that European red deer (*Cervus elaphus elaphus*) are susceptible to intra-cerebral (i/c) challenge with BSE positive cattle brain pool material resulting in clinical neurological disease and weight loss by 794–1290 days and the clinical signs are indistinguishable to those reported in deer with CWD. Spongiform changes typical of TSE infections were present in brain and accumulation of the disease-associated abnormal prion protein (PrP^d) was present in the central and peripheral nervous systems, but not in lymphoid or other tissues. Western immunoblot analysis of brain material showed a similar glycosylation pattern to that of BSE derived from infected cattle and experimentally infected sheep with respect to protease-resistant PrP isoforms. However, the di-, mono- and unglycosylated bands migrated significantly ($p < 0.001$) further in the samples from the clinically affected deer when compared to BSE infected brains of cattle and sheep.

Conclusion: This study shows that deer are susceptible to BSE by intra-cerebral inoculation and display clinical signs and vacuolar pathology that are similar to those of CWD. These findings highlight the importance of preventing the spread to Europe of CWD from North America as this may necessitate even more extensive testing of animal tissues destined for human consumption within the EU. Although the absence of PrP^d in lymphoid and other non-neurological tissues potentially limits the risk of transmission to humans, the replication of TSE agents in peripheral tissues following intra-cerebral challenge is often limited. Thus the assessment of risk posed by cervine BSE as a human pathogen or for environmental contamination should await the outcome of ongoing oral challenge experiments.

Background

Bovine spongiform encephalopathy (BSE), which affects cattle and several other food animal species [1,2], belongs to the transmissible spongiform encephalopathy (TSE) group of fatal neurodegenerative diseases affecting humans and animals [3,4] and can be transmitted within and between species by ingestion or parenteral inoculation [5]. TSEs which include, amongst others, scrapie in sheep and goats, sporadic Creutzfeldt-Jakob disease (CJD) in humans and chronic wasting disease (CWD) of deer [6] are all characterised by long incubation periods leading to clinical neurological manifestations. The pathological changes can usually be linked with the conversion of the normal host-encoded membrane associated prion protein (PrP^{C}) to abnormal disease-associated isoforms (PrP^{d}) and their accumulation in the nervous system and, depending on the host species and the TSE agent involved, the lymphoreticular system [7]. Detection of PrP^{d} in tissues by various laboratory methods is the current approach for a definitive diagnosis of any TSE [8].

Ten years after the first report of BSE in cattle [9] variant Creutzfeldt-Jakob disease (vCJD) was diagnosed in humans in the UK [10] and is invariably fatal [11]. Ingestion of BSE contaminated bovine products was subsequently linked to the development of vCJD [12] resulting in greatly intensified surveillance and research in both humans and food animals [13].

During the 1980s UK farmed deer were fed relatively large amounts of proprietary concentrate feed containing ruminant derived meat and bone meal (MBM) due to the market demand for well grown breeding stock. Additionally, some free ranging estate deer, park deer and cervids of many different species kept in UK zoological collections were also fed similar proprietary concentrate feed (*pers com.* Dr. T.J. Fletcher). It is highly unlikely that these concentrates were free from BSE contaminated MBM suggesting that UK deer have been exposed to infectious material. Surveys of several thousand deer brains have so far revealed no evidence of any sub-clinical TSE infection in deer in Europe. This is despite the presence in North America of CWD, a highly infectious TSE which specifically affects deer including the elk (*Cervus elaphus nelsoni*), a sub-species of European red deer, and is currently the only TSE maintained in free ranging populations [14]. However, the European surveys have either not concentrated on farmed populations, been regionally restricted, had limited numbers or have not specifically targeted clinical neurological suspects [15-20].

Results

In the present study 6 European red deer (*Cervus elaphus elaphus*), the most commonly farmed cervid species in the UK, were challenged intracerebrally (i/c) with BSE positive bovine brain material and a further 2 deer received sterile saline by the same route. All deer on the study were methionine homozygous at codon 132 of the cervid prion protein [21] equivalent to codon 129 in humans [22]. All six BSE-inoculated animals developed a variable range of neurological clinical signs including ataxia, anorexia, circling and apparent blindness along with failure of seasonal change of coat, weight loss and 'panic attacks' between 794–1300 days post-challenge (Table 1). The panic attacks comprised various episodes of mania of differing severity and duration, which in the first and last animals to develop clinical signs resulted in self trauma requiring euthanasia on welfare grounds. Five of the affected deer were euthanased and one (deer No. 5) died of inhalation pneumonia. With the exception of very little intra-thoracic or abdominal adipose tissue, which was apparent in all animals, gross lesions were present only in the animal that died of inhalation pneumonia. Severe consolidation of the right apical lung lobe was present with red/green discolouration, the tissue was very friable and a low-viscosity purulent material oozed from the cut surface. Also, blood was present in the caecum of this animal.

Spongiform change characterized by vacuolation of both neuronal perikarya and grey matter neuropil was prominent in the brains of all six clinically affected deer (Figure 1). Optically empty, round or oval vacuoles were present in neuropil while neuronal perikaryonal vacuoles were sometimes loculated and sometimes contained membranous debris. The appearance and distribution of the vacuoles are thus indistinguishable from those of other classical TSEs, including CWD. Lesions predominantly affected the brain stem, thalamus and striate body, as well as the molecular layer of the cerebellum and cerebrocortical layers V and VI.

Immunohistochemical labelling of PrP^{d}

Accumulation of PrP^{d} in the BSE infected deer was restricted to the central and peripheral nervous systems, including all levels of the spinal cord, the autonomic ganglia, peripheral and cranial nerves, the enteric nervous system and the sensory retina. Essentially the same neuroanatomic pattern and types of PrP^{d} accumulation were found with each of the two antibodies (F99 and Bar 224) that were used. PrP^{d} deposits were more inconsistent and less intense in the first deer to show clinical signs compared with the other five, possibly due to its shorter incubation period (794 days, Table 1). PrP^{d} was found to accumulate in all anatomical areas of the brain though levels were relatively lower in the cerebral cortex. Typically, only grey matter was involved, with the most prominent accumulations being of the fine particulate type, with some more coarse and linear deposits in severely affected areas (Figure 2). Granular deposits of PrP^{d} within

Table 1: Clinical parameters of BSE infection in deer.

Clinical parameters	Deer identification							
	1	2	3	4	5	6	7	8
Incubation period (challenge-PME date) (days)	794	930	996	996	1060	1290	1017	1290
Clinical signs duration (days)	21	100	105	77	85	22	N/A	N/A
Weight loss (peak weight to PME [kg])	12.9	20.0	19.8	19.1	24.3	11.5	9.6	0.5
Weight loss (as % of peak weight)	13.2	27.3	20.4	21.8	39.2	11.9	10.3	0.6
Ataxia	+	++	++	++	+	-	-	-
Anorexia	-	+	+	+	++	-	-	-
Circling	-	+	++	++	-	-	-	-
Failure of seasonal change of coat	-	+	+	+	-	-	-	-
Apparent blindness	-	-	+	+	-	-	-	-
Panic attacks	+++	++	+	-	-	++	-	-
Inhalation pneumonia	-	-	-	-	+++	-	-	-

Deer 1–6 challenged i/c with BSE agent in chronological order of developing clinical signs. Deer 7 and 8 challenged i/c with sterile saline – negative control animals.PME = post-mortem examination N/A = not applicable-negative, + mild, ++ moderate, +++ severe

the cytoplasm of neurons were prominent, especially throughout the brainstem and thalamic nuclei, though they were less conspicuous in the striate body and cerebral cortex. In the cerebellum, accumulation of intra-neuronal PrP^d was conspicuous in the deep nuclei and, distinctly, in the Golgi neurons of the granular layer, but not in the Purkinje cells (Figure 3). Intracellular deposits of PrP^d were also noticeable in astrocytes and microglial cells in areas of heavy neuropil involvement. In the cerebral grey matter, the PrP^d around glial cells produced a diffuse stellate pattern of accumulation. Very little PrP^d accumulation was present around blood vessels in cerebral white matter or as intra-astrocytic granules in the cerebellum. PrP^d was not detected in lymphoreticular tissues, skeletal muscle, kidneys or any of the other organs examined.

Western immunoblotting

Western immunoblot analysis of individual samples of cerebellum from the 6 clinically affected deer using antibody L42 after treatment with proteinase K showed a glycosylation pattern of protease-resistant PrP (PrP^{res}) in

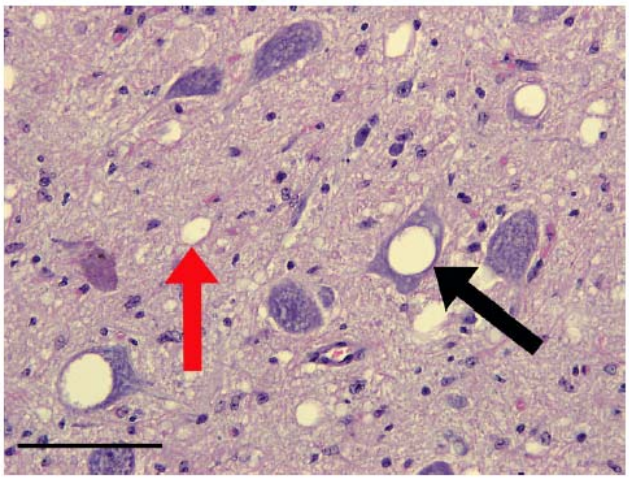

Figure 1
Spongiform change in the obex of the brain from a clinically affected BSE challenged deer. Note the optically empty vacuoles in both neuronal perikarya (black arrows), occasionally containing membranous debris, and also the neuropil (red arrows). Haematoxylin and eosin. Bar = 50 μm.

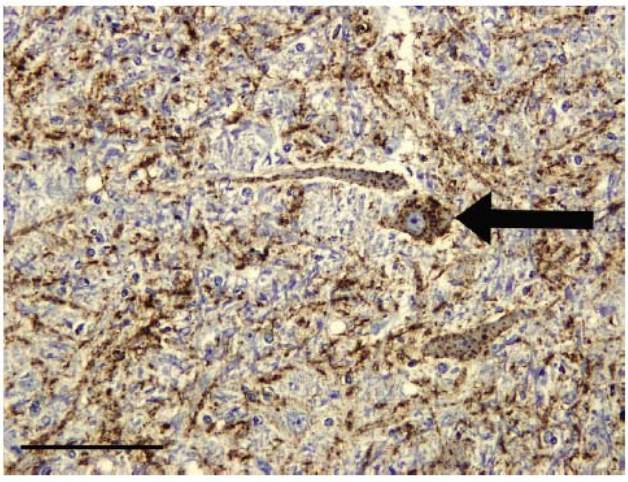

Figure 2
Patterns of PrP^d accumulation in the brain of clinically affected BSE challenged deer. PrP^d immunohistochemical labelling of the vestibular nuclei using Bar 224 antibody shows that the neuropil has severe particulate and linear deposits of PrP^d accumulation (brown pigment denotes PrP^d). There is also marked intraneuronal labelling present (arrow). Bar = 100 μm.

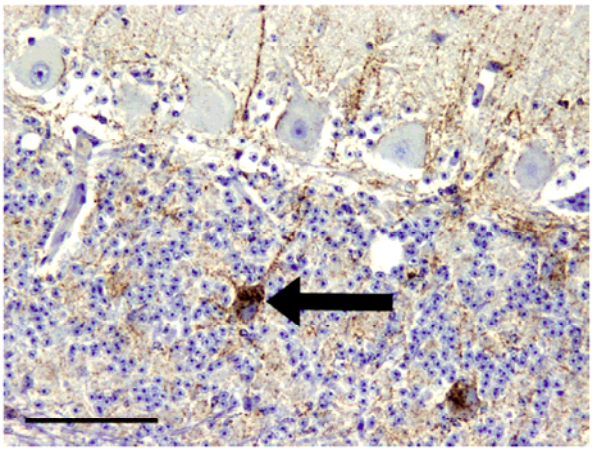

Figure 3
Intraneural PrP ^{d} accumulation in Golgi neurons of clinically affected BSE challenged deer. PrP^{d} immunohistochemical labelling of the cerebellar cortex using Bar 224 antibody shows accumulation in granular and molecular layers. There is prominent intraneuronal PrP^{d} accumulation in Golgi neurons (arrow). Linear forms of accumulation are also present, one of which is associated with a Golgi neuron cell body (brown pigment denotes PrP^{d}). Bar = 100 μm.

which the di-glycosyl fraction predominated (Figure 4 and Table 2) with some minor variations in labelling intensity between animals. The deer di-, mono- and unglycosylated bands migrated significantly ($p < 0.001$) further than the corresponding bands in both the cattle and experimental ovine BSE samples, which in turn migrated significantly more than ovine scrapie. None of the BSE-infected samples, irrespective of the species of origin (bovid, ovid or cervid), reacted with antibody P4 in an identical Western immunoblot but the sample of scrapie infected ovine brain did (data not shown). The lack of labelling by antibody P4 is a recognised method of differentiation between ovine BSE and scrapie derived PrP^{res} [23]. Additionally, all cervid samples did label with antibody P4 in the absence of proteinase K treatment indicating that PrP^{C} is detected by this method in all species examined (data not shown). This absence of detection of cervid PrP^{res} after proteinase K treatment by the P4 antibody is consistent with immunoblot characteristics of other experimental ruminant BSE infections and some experimental sheep scrapie sources such as CH1641 [24].

Discussion

This is the first report of the successful experimental transmission of BSE to any species of deer and the first report of any TSE in European deer. European red deer were chosen because in the UK they are the cervid species most commonly given supplementary proprietary feed under both farmed or free-ranging estate management and therefore are the species most likely to have been exposed to BSE contaminated MBM prior to its exclusion from animal feed. Additionally, they are very closely related to the North American elk which is susceptible to CWD [14]. The resultant clinical signs in the BSE challenged red deer were similar to those reported in both naturally acquired and experimental CWD in cervids in North America, including death due to secondary inhalation pneumonia [14]. As such, we believe it would not be possible to differentiate BSE, if it occurred naturally, from CWD in deer by routine clinical examination in the field.

The vacuolation pattern found in the brains of the BSE challenged red deer is similar to that reported in cattle with BSE except that the mesencephalon appeared to be more affected in the latter [13]. Sheep orally infected with BSE also developed a similar vacuolation pattern which favours the brainstem [25] as do mule deer (*Odocoileus hemionus*) and elk with clinical CWD [26] such that the patterns of vacuolation in the brain are unlikely to provide a simple method to distinguish between individual BSE or CWD infections in deer.

PrP^{d} labelling by IHC in tissues from the clinically affected i/c BSE challenged red deer showed it to be restricted to the central and peripheral nervous systems. This suggests an incubation-period related centrifugal spread of infectivity from the brain and associated PrP^{d} accumulation through the nervous system. Overall, the immunohistochemical features of BSE in red deer after i/c transmission are similar to those reported for cattle, sheep or goats naturally or experimentally infected with BSE, in which there is prominent intra-cellular PrP^{d} accumulation as well as widespread particulate labelling in areas of grey matter neuropil, including that surrounding the soma of neurons and their processes along with linear and multifocal stellate reticular forms associated with glial cells [13]. Follow-

Table 2: Mean relative percentage intensities of labelled PrP^{res} di-, mono- and aglycosyl bands from Western blots

Immunolabelled band	Natural Scrapie	Ovine BSE	Bovine BSE	Deer 1	Deer 2	Deer 3	Deer 4	Deer 5	Deer 6
diglycosyl	44	54	62	57	50	51	52	46	61
monoglycosyl	36	31	26	32	35	34	33	35	32
aglycosyl	20	15	12	11	15	16	15	19	7

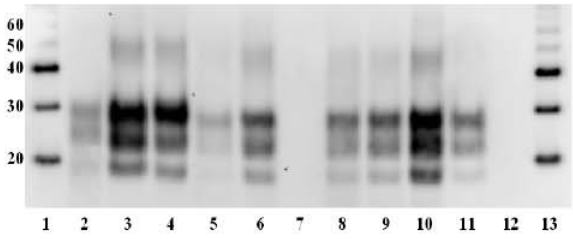

Figure 4
Glycosylation patterns of PrP^{res}. Western immunoblot of brain samples after treatment with Proteinase-K and using antibody L42 (R-biopharm, diluted 1/2000) to label proteinase resistant PrP. Note significantly ($p < 0.001$) greater migration of di-, mono- and unglycosylated bands in all 6 clinically affected deer (lanes 5, 6, 8–11) compared to sheep scrapie, experimental ovine BSE and cattle BSE (lanes 2–4 respectively). Also, lack of labelling of protease resistant PrP in negative control deer (lanes 7 and 12). Lane 1 – molecular weight markers (kDa), lane 2 – sheep scrapie, lane 3 – experimental ovine BSE, lane 4 – BSE (inoculum), lane 5 – clinically affected deer 1, lane 6 – clinically affected deer 2, lane 7 – negative control (deer 7 in table 1), lane 8 – clinically affected deer 3, lane 9 – clinically affected deer 4, lane 10 – clinically affected deer 5, lane 11 – clinically affected deer 6, lane 12 – negative control (deer 8 in table 1), lane 13 – molecular weight markers (kDa).

ing high dose oral challenge of cattle several studies have also shown an inconsistent presence of BSE infectivity or PrP^{d} labelling in some peripheral nerves, Peyer's patches and tonsils [27,28]. However, the infectivity in both neural and non-neural tissues from BSE affected deer following oral challenge is yet to be determined. Sheep that have been challenged orally with bovine derived BSE differ from both cattle and i/c challenged red deer in that they show extensive labelling of PrP^{d} in lymphoid tissues in addition to the peripheral and central nervous systems [25] similar to deer with CWD [14].

In contrast to our findings in BSE infected red deer where prominent intra-neuronal labelling of PrP^{d} was found, intra-neuronal deposits are reportedly rare in CWD affected animals and when present are mild and scattered within and between neurones of different nuclei; mainly in the brainstem [29] and the cerebellar deep nuclei [30]. However, detailed descriptions of the neuropathology of different CWD sources are few and it is not clear whether CWD consists of a single or many TSE strains. Whether BSE in deer can be readily distinguished by IHC epitope mapping [31] or PrP^{d} profile [32] will require additional study.

Differentiation of BSE from CWD in deer would appear possible by IHC as PrP^{d} is restricted to the nervous system in BSE yet present in the lymphoid tissue in CWD. However, the red deer in this study were experimentally challenged by the i/c route and we cannot be certain of the distribution of PrP^{d} in deer exposed to natural, presumably oral, challenge with BSE.

Western immunoblot analysis of the brains from the 6 affected red deer showed that the PrP^{res} pattern of i/c cervid BSE has similarities to cattle BSE including a relatively fast migrating aglycosyl band and predominance of the diglycosyl band (Figure 4). The greater intensity of labelling of deer 5 may be due to it dying, and therefore reaching a terminal end point, rather than a clinical end point where animals were euthanased at an agreed severity of clinical signs, as occurred in the previous four affected deer. Also, deer 5 was the only female in the BSE i/c challenge group, however, we are uncertain if this had any effect on PrP^{res} glycoprofile or levels of accumulation. The significantly ($p < 0.001$) greater migration of all three protease resistant bands (di-, mono- and unglycosylated) in the red deer samples compared to the cattle and sheep BSE suggests some host adaptation of BSE PrP^{res} propagated within the red deer nervous system. The altered biochemical properties suggests the possibility of alternative truncation of the PrP molecule within deer compared with the bovine or ovine host.

Conclusion

This study shows that European red deer are susceptible to i/c challenge with the BSE agent resulting in a disease that is clinically indistinguishable by routine clinical examination from that reported for CWD [14]. Thus strong measures to prevent the spread of CWD to Europe are essential as even small numbers of CWD cases could result in the need for extensive testing of deer tissues destined for human consumption.

The susceptibility of UK red deer to natural, presumably oral, exposure is still uncertain. The absence of PrP^{d} in lymphoid tissues in the present work might appear to limit the risk to humans of infection from venison and other non-neuronal edible deer tissues and also limits the maintenance of natural infection in the environment. However, the susceptibility of peripheral tissues to infection cannot be ascertained from i/c challenge and must await the outcome of parallel oral challenge experiments.

Methods

Animal procedures

Eight European red deer calves were housed at 1–2 days old, initially hand reared with artificial milk and then given *ad-libitum* access to water, hay and proprietary concentrated feed when weaned. All experimental protocols

were approved by the Moredun Research Institute Animal Experiments Ethical Committee and authorised under the UK Animals (Scientific Procedures) Act 1986. One half ml of a 10% solution of BSE brain material (BBP12/92, which consisted of a pool of 5 BSE positive bovine brains with comparative titres of $10^{6.0}$ cattle [i/c] units LD 50/g and $10^{3.3}$ mouse [i/c/intra peritoneal] units LD 50/g, VLA-Weybridge, UK) diluted in sterile normal (0.9%) saline and containing 1.25 mg/ml ampicillin was inoculated into the right cerebral hemisphere under general anaesthesia (n = 6, 5 castrated males and 1 female, 10–12 months old). Control animals (n = 2, female, 10–12 months old) underwent an identical procedure with sterile normal saline containing 1.25 mg/ml ampicillin. All animals were observed daily for clinical signs of disease and weighed monthly.

Genotyping

Genotyping was performed either from blood samples taken from live deer into vacutainers containing EDTA (BD Bioscience, Erembodegem, Belgium) or from frozen brain material collected post-mortem. DNA was extracted from blood using the CST Genomic DNA Purification Kit (Charge Switch™ Technology, DNA Research Innovations, Ltd., Sittingbourne, UK) and from frozen brain tissue using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. PCR amplification of genomic DNA was carried out using PCR primers C.e. 19fwd 5' ATT TTG CAG ATA AGT CAT C 3' and C.e. 778rev 5' AGA AGA TAA TGA AAA CAG GAA G 3' [21]. The PCR reaction was carried out using a hot start of 95°C, 15 min then 10 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec then 30 cycles of 95°C for 30 sec, 59°C for 30 sec, 72°C for 59 sec. Amplified samples were then sequenced by the dideoxy chain termination method using ET terminator chemistry using a MegaBACE 500 instrument and Cimarron 3.12 basecaller (GE Healthcare, Amersham plc., Buckinghamshire, UK).

Post-mortem examination

The 6 BSE challenged deer (5 males and 1 female), and 2 clinically normal negative control deer (both female) were subjected to full post-mortem examination and an extensive range of tissue samples taken from: 1) the nervous system (brain and pituitary, cervical, thoracic and lumbar spinal cord with associated dorsal root ganglia, the trigeminal, nodose, stellate and cranial mesenteric ganglia, the vagus nerve, the sympathetic chain, the second muscular branch of the sciatic nerve and the eye), 2) the lymphoreticular system (third eyelid, submandibular, retropharyngeal, prescapular, mediastinal, popliteal and proximal and distal jejunal lymph nodes, the palatine tonsil and spleen), gastro-intestinal tract (oesophagus, omasum, abomasum, duodenum, jejunum, distal ileum and colon all with their associated Payer's patches and the caecum), 3) body fluids (cerebrospinal fluid, blood serum, blood buffy coat and urine), and 4) other tissues (skin of the ventral lip, inter-ventricular septum of the heart, left caudal lung lobe, liver, kidney, semitendinosus muscle and uterus and mammary gland if present). All samples were fixed in 10% neutral buffered formalin and/or frozen and stored at -80°C. Fixed samples were post-fixed in fresh 10% neutral buffered formalin then routinely processed for embedding in paraffin wax. Four μm thick sections were cut and mounted on glass slides (Superfrost Plus; Menzel-Gläser, Braunschweig, Germany) and either stained with haematoxylin and eosin or subjected to IHC.

Immunohistochemical localisation of PrP^d

This was performed as previously described [33]. Briefly, antigen retrieval included immersion in 98% formic acid for 5 minutes followed by autoclaving for 30 minutes at 121°C in 0.2% citrate buffer. Endogenous peroxidase activity was quenched with 0.9% (v/v) hydrogen peroxide in distilled water for 20 minutes and to block reactivity of non-specific tissue antigens sections were incubated in 20% normal horse serum for 1 hour. Following this, incubation with the primary antibody was carried out overnight at 27°C. The subsequent steps of the IHC protocol were performed by a commercial immunoperoxidase technique (Vector-elite ABC kit; Vector Laboratories, Peterborough, UK) at the end of which sections were immersed in 0.5% copper sulphate to enhance any immunoperoxidase colour reaction and finally counterstained with Mayer's haematoxylin prior to routine dehydration and mounting. Primary antibodies were either F99, clone 97.6.1 (VMRD Inc., Pullman, USA), which binds to amino acid sequence 220–225 of human PrP or BAR224 (CEA, Saclay, France) which recognizes amino acid sequence 141–147. Both of these antibodies have wide inter-species reactivity.

Western immunoblot analysis of brain samples

Samples of frozen cerebellum were allowed to warm to room temperature and homogenised at 10% (w/v) in lysis buffer (tris-buffered saline [TBS] pH 7.4, 0.5% Na-deoxycholate, 0.5% NP-40) with a Fast Prep instrument (Q-biogene, Cambridge, UK) using 1 cycle of 6.0 ms/40 sec. Lysates were held at 4°C for 2 h then centrifuged 100 × g for 1 minute and aspirated to new tubes avoiding any remaining tissue debris. 100 μl of lysate was treated with proteinase K solution (50 μg/ml) for 1 hour at 37°C with shaking. Digestion was terminated by adding Pefabloc SC (Roche Diagnostics, Burgess Hill, West Sussex, UK) to a concentration of 1 mM. Samples were then centrifuged at 20,000 × g for 1 hour at 10°C. Supernatants were discarded and pellets resuspended in 45 μl 2 × SB (Invitrogen, Paisley, UK) containing 5 μl of 10 × sample reducing agent (Invitrogen, Paisley, UK). Samples were heated at

100°C for 5 minutes then briefly centrifuged for 5 seconds at 20,000 × g. SDS-PAGE was carried out on 5–20 μl of sample on 12% Bis-Tris NuPAGE gels (Invitrogen, Paisley, UK) at 150 V for 1 hour. One mg brain equivalent was loaded for each sample except for BSE, experimental ovine BSE and deer 1 all of which had 1.5 mg loaded to enable visible staining on the resultant Western blots. Proteins were electrotransferred onto Hybond P PVDF membrane (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) at 30 V for 1 hour. Non-specific antigen binding on the membrane was blocked by soaking in 5% non-fat milk/TBS with 0.1% $Tween_{20}$ (Sigma Chemical Company, Poole, Dorset, UK) and probed with either antibody L42 (R-biopharm, Darmstadt, Germany, diluted 1:2000), which binds to amino acid sequence of 145–163 of ovine PrP, or antibody P4 (R-biopharm, Darmstadt, Germany, diluted 1:2000), which binds to amino acids WGQGGSH (sequence 93–99 of ovine PrP) [34]. A Western immunoblot was also prepared from identical samples with the omission of the proteinase K digestion step and labelled with antibody P4. Signal was detected using Super Signal West Fempto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and a Kodak IS440 image station (Labtech International Ltd., Lewes, UK). The relative intensities of the di-, mono- and aglycosyl bands of PrP^{res} were determined by scanning the Western blot image using Kodak 1D Image Analysis Software.

Statistical analysis of Western immunoblot

Each of the three relevant bands (di-, mono-, and unglycosylated) positive for protease resistant PrP by labelling with antibody L42 on Western immunoblots were analysed separately for distance migrated using a linear mixed model with the type of sample included as a fixed effect in the model and blot included as a random effect in the model. The parameters in the model were estimated using the REML directive in Genstat 10th edition. Using this model accounts for the differences in mean levels between blots for each type of band separately (as opposed to subtracting off a constant amount from all the readings on one blot to allow for differences between them). The three bands in a single column are not independent of each other and so the results from the three separate analyses are likely to be correlated. Data were available from five replicate Western immunoblots each containing samples representing scrapie, experimental ovine BSE, bovine BSE and the six BSE challenged clinically affected deer (only five samples for i/c challenged deer were present on three of the blots, deer 6 was absent) and the two negative control animals (which did not label for protease resistant PrP). Samples were considered to be significant at $p \leq 0.05$.

Authors' contributions

MJ, LG and HWR designed the study and the project was managed by SM and MPD. Dosing, weighing, monitoring and evaluation of clinical endpoint MPD, PS, JF, SH & HWR. Necropsies: MPD, SS, LG, MJ, FC, PS, JF & SH. Pathology SM, MPD, MJ, LG & SS. Biochemistry PS & SH. Collated the results and wrote the paper MPD, SM, MJ & PS.

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Annex B

Immunohistochemical and biochemical characteristics of BSE and CWD in experimentally infected European red deer (*Cervus elaphus elaphus*)

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Abstract

Background: The cause of the bovine spongiform encephalopathy (BSE) epidemic in the United Kingdom (UK) was the inclusion of contaminated meat and bone meal in the protein rations fed to cattle. Those rations were not restricted to cattle but were also fed to other livestock including farmed and free living deer. Although there are no reported cases to date of natural BSE in European deer, BSE has been shown to be naturally or experimentally transmissible to a wide range of different ungulate species. Moreover, several species of North America's cervids are highly susceptible to chronic wasting disease (CWD), a transmissible spongiform encephalopathy (TSE) that has become endemic. Should BSE infection have been introduced into the UK deer population, the CWD precedent could suggest that there is a danger for spread and maintenance of the disease in both free living and captive UK deer populations. This study compares the immunohistochemical and biochemical characteristics of BSE and CWD in experimentally-infected European red deer (*Cervus elpahus elaphus*).

Results: After intracerebral or alimentary challenge, BSE in red deer more closely resembled natural infection in cattle rather than experimental BSE in small ruminants, due to the lack of accumulation of abnormal PrP in lymphoid tissues. In this respect it was different from CWD, and although the neuropathological features of both diseases were similar, BSE could be clearly differentiated from CWD by immunohistochemical and Western blotting methods currently in routine use.

Conclusion: Red deer are susceptible to both BSE and CWD infection, but the resulting disease phenotypes are distinct and clearly distinguishable.

Background

Bovine spongiform encephalopathy (BSE) is one of a group of transmissible spongiform encephalopathies (TSEs), which include Creutzfeldt-Jakob disease in humans, chronic wasting disease (CWD) in deer and scrapie in sheep and goats. These invariably fatal neurodegenerative disorders typically show lesions such as spongiform change, gliosis and deposition of the disease-associated form (PrP^d) of the normal cellular prion protein (PrP^c).

Contaminated meat and bone meal (MBM) within concentrated rations fed to cattle was the cause of the BSE epidemic in the United Kingdom (UK [1]) and elsewhere in Europe. Exotic ungulates were also exposed to this material resulting in cases of BSE in zoological collections [2,3], and it is likely that cervids in these collections were also exposed [4]. Similarly, contaminated rations could have also been fed to farmed and free ranging deer, the latter as a winter supplement. In March 2007, the European Union adopted a Commission Decision calling for a survey for TSEs in cervid populations, and monitoring of farmed and wild red deer began in the UK. Despite there not being any reported cases so far, the possibility that BSE infection occurred in deer prior to MBM bans cannot be ruled out as, on the other hand, several deer species are affected by CWD. One of those species is the elk or wapiti (*Cervus elaphus nelsoni*), which is closely related to the red deer (*Cervus elaphus elaphus*), the most frequently farmed cervid in the UK. Unlike other TSEs, CWD is the only TSE that is maintained in free-ranging animals with low population densities (2–3 deer per square mile), and surveillance data suggests an increasing prevalence of infection [5]. It is highly transmissible by natural routes of infection, so that the prevalence of infection can reach 100% in farmed herds [6], perhaps also influenced by the fact that, unlike sheep with scrapie, deer appear not to have any absolute genetic resistance to CWD [7].

Should BSE infection have been introduced to the UK red deer population, both the stability of the BSE agent through the species barriers and the high transmissibility of CWD, could account for spread and maintenance of BSE in free-living and captive UK deer. Due to the potential risk for human health from the consumption of contaminated deer products, it was deemed appropriate to determine the susceptibility or resistance of UK red deer to BSE and, if susceptible, to characterize the resulting disease phenotype in comparison with that of CWD in the same subspecies.

We previously described the susceptibility of UK red deer to BSE infection by intracerebral injection [8], and the present paper deals mainly with the comparative immunohistochemical (IHC) and Western blot (WB) features of experimental BSE and CWD infections in red deer. In addition, proof of concept is given that red deer are also susceptible to BSE by the oral route, albeit at high dose and apparently with a low attack rate and extended incubation period. In doing the experiments described here, we have also accrued a bank of positive control material to help future research and to be used as reference in the surveillance for BSE in red deer.

Methods

Experimental procedures

BSE experiments

Six one year-old red deer were intra-cerebrally (i.c.) inoculated with 0.5 ml of a 10% homogenate of a brain pool of five BSE-positive bovine brains (BBP12/92); this experiment has already been described [8]. A further six red deer aged 4 weeks were orally dosed via stomach tube with 25 g of another cattle BSE brain pool homogenate containing $10^{3.5}$ RIII mouse (i.c./intraperiotoneal) units ID50/g; as with the BSE inoculum for i.c. challenge, this one also was provided by the TSE archive at VLA-Weybridge. Two and four negative control animals underwent identical procedures to the i.c and orally dosed deer, respectively, with sterile normal saline instead of brain homogenate. All experimental animal procedures were approved by the Moredun Research Institute Animal Experiments Ethical Committee and authorised under the UK Animals (Scientific Procedures) Act 1986.

CWD experiment

This was carried out by the Canadian Food Inspection Agency (Ottawa, Canada). Four European red deer were challenged with 5 g of a pooled brain homogenate from four elk with clinical CWD. The inoculum was administered in three doses three days apart and although it was not titrated, it gave an optical density reading of >3.5 (upper limit of measurement) in the Bio-Rad TsSeE test.

Disease monitoring, post-mortems and tissue sampling

Deer challenged with BSE were clinically monitored daily and weighed monthly, until they reached a clinical end point established at the beginning of the experiment, as previously described [8]. After discussion and agreement, a similar approach was applied to CWD inoculated red deer. Biopsies of rectal mucosa were sequentially taken during the incubation period of both BSE and CWD inoculated animals; rectal biopsies may be used for pre-clinical diagnosis of TSEs in sheep [9] and in deer [10]. Deer were killed by barbiturate overdose and exsanguination. At post-mortem, an extensive range of tissue samples was taken and fixed in 10% neutral buffered formalin or frozen and stored at -80°C.

Histopathology and IHC

Fixed samples were post-fixed in fresh 10% neutral buffered formalin and routinely processed for embedding in paraffin wax and light microscope examinations, either

after staining with haematoxylin and eosin or immunolabelling for PrP^{d}. The tissues examined by IHC from BSE inoculated deer have been described in detail previously [8], and included samples from the central and peripheral nervous systems, lymphoreticular system (LRS), and several other tissues. From CWD inoculated deer, eight brain areas and the medial retropharyngeal lymph node were available for IHC examinations.

The IHC method has been detailed elsewhere [11,12]. Briefly, tissue sections were subjected to antigen retrieval, peroxidase quenching and blocking of non-specific antigens prior to incubation with the primary antibody. This was carried out overnight at 27°C, and the subsequent steps of the IHC protocol were performed by a commercial immunoperoxidase technique (Vector-elite ABC kit; Vector Laboratories, Peterborough, UK) and finally counterstained with Mayer's haematoxylin. PrP primary antibodies used were BAR224 (CEA, Saclay, France), which recognizes amino acid residues (aa) 141–147 of ovine PrP [13], and 12B2 (CIDC, Lelystad, The Netherlands), recognizing aa 93–97 of ovine PrP [14]. Both antibodies have wide inter-species reactivity and were selected as the most specific and sensitive from a panel of antibodies tested against CWD positive and negative deer control tissues at the outset of the study.

Western blot

Samples of medulla oblongata from BSE and CWD inoculated deer developing clinical disease and from non-inoculated controls were processed using the BioRad TeSeE universal WB, following the kit instructions. Briefly, 350 mg of tissue were ribolysed, purified, treated with proteinase K, and PrP^{res}-concentrated. After heating, 20 mg equivalent of samples were loaded in duplicate lanes onto pre-cast 12% bis-tris gels (Bio-Rad laboratories Ltd. Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX.), and subjected to electrophoresis. The proteins were then transferred onto PVDF membranes, blocked (Bio-Rad blocking solution) and probed for 30 mins at room temperature with each of a panel of five antibodies: 6H4 (aa 156–164 [15]), F99 (aa 229–232 [16]), Sha31 (aa 156–163 [17]), P4 (aa 93–99 [18]), and 12B2. After washing, the membranes were incubated with the secondary antibody at room temperature, and with ECL substrate (Amersham Biosciences UK limited) for 45 s – 1 min, and the signal was detected with the Fluor-S MultiImager (Bio-Rad, Maxted Road Hemel Hempstead Hertfordshire HP2 7DX, UK).

The blots were assessed for molecular weights and proportions of the three PrP^{res} bands, and for the affinities for each PrP antibody. For comparison, brain samples from CWD positive and negative elk, BSE positive cattle and scrapie positive sheep were used.

Discriminatory and scoring methods

The IHC "epitope mapping" and "PrP^{d} profiling", and the discriminatory WB allow differentiation between sheep BSE and natural and experimental scrapie in sheep and in other species. The IHC "epitope mapping" and the discriminatory WB are based on the availability of specific epitopes on PrP^{d} subjected to enzymatic attack "*in vivo*" (in intracellular lysosomes) and "*in vitro*" (by protease digestion), respectively [19,20]. It has been hypothesized that in experimental ovine BSE, epitopes between amino acids 93 and 97 of the PrP sequence are absent after enzymatic digestion, as truncation of the protein occurs further towards the C terminus, and therefore, these truncated forms of PrP^{d} are not labelled by 12B2 or P4. In contrast, in CWD and scrapie, the PrP^{d} molecule is truncated further towards the N terminus and the labelling with those antibodies is maintained after "*in vivo*" or "*in vitro*" digestion. "PrP^{d} profiling" is the most sophisticated technique for IHC strain differentiation: using a single antibody directed at the globular or C-terminal domains of the protein, this approach entails the subjective scoring (0–3) of different PrP^{d} types (intra-cellular, cell membrane bound and extracellular) across different areas of the brain to produce a graphic profile, which is representative of the IHC phenotype [11,12].

Results

Clinical disease

All six deer challenged i.c. with BSE developed clinical disease between 794 and 1260 days post-inoculation with a mean incubation period of 1027 days. A detailed description of the clinical signs was provided in an earlier report [8]. Briefly, affected deer showed variable degrees of ataxia, anorexia, circling and apparent blindness, together with failure of seasonal change of coat, weight loss and 'panic attacks'. In addition, one of six red deer orally dosed with BSE developed clinical disease 1740 days after challenge, and this animal presented with a short clinical duration of two days; the other five deer from this group remain healthy at the time of writing (65 months after challenge). Sequential rectal biopsies taken at five different time points from orally and i.c. inoculated deer were negative for PrP^{d}.

All four deer orally challenged with CWD started to show behavioural changes between 577 and 586 days post challenge; these progressed to definite neurological disease between 742 and 760 days post-challenge (Table 1). Clinical signs were similar to the BSE challenged deer and included nervousness, weight loss, excessive salivation, roughness of coat, and progressive ataxia. All these CWD-inoculated deer showed PrP^{d} accumulation in the secondary follicles of rectal biopsies taken at 7 months post-infection.

Table 1: Experimental design and clinical data.

Inocula	Route		Deer					
			1	2	3	4	5	6
BSE	I.C	I.P	794	930	996	996	1060	1260
		C.D	21	100	105	77	85	22
	Oral	I.P	1740					
		C.D	2					
CWD	Oral	I.P	577	579	579	586		
		C.D	165	167	167	174		

Deer numbers, routes of infection, incubation period (I.P) and clinical duration (C.D) in days.

Histopathology and IHC

Spongy change

In haematoxylin and eosin stained brain sections, spongiform change was widespread in all seven deer that developed clinical signs after BSE challenge regardless of the route. Vacuolation was seen in the neuronal perikaryon and neuropil of the brainstem, the molecular layer of the cerebellum, the thalamus, and the layers V and VI of the cerebral cortex (Fig 1). Spongy change was also observed in the same brain areas of the CWD-challenged deer; in these, intracytoplasmic vacuoles within neurones, although present, were less prominent than in the BSE challenged group. Otherwise no differences in pathological features were identified between the two groups of deer. Overall, spongiform changes and their distribution were similar to those reported in cattle with BSE [21], in sheep orally infected with BSE [22], and in CWD infected mule deer and elk [23].

Accumulation of PrPd in the brain. Widespread diffuse particulate labelling of the neuropil and intense intra-neuronal accumulations of PrP^d were observed throughout the brains of BSE affected deer in sections labelled with BAR224 antibody. Peri-neuronal labelling was prominent in the dorsal motor nucleus of the vagus nerve (DMNV) and in the *corpus striatum*. Intraneuronal granular accumulations were prominent in nuclei of the *medulla oblongata*, such as the accessory cuneate, spinal trigeminal and posterior olivary, but were also present elsewhere in the brain with the exception of Purkinje cells. Notably, there was also intense labelling of the Golgi neurones in the granular cell layer of the cerebellum (Fig 2), which has not been described in sheep or goat scrapie and which was absent in the CWD infected deer. Slight differences in topographical distribution of PrP^d accumulation were observed between the BSE affected deer: while five of the i.c. inoculated animals showed abundant PrP^d deposits in forebrain areas, the single orally-challenged and one of the i.c. inoculated animals accumulated PrP^d predominantly in the brainstem and cerebellum, and very little elsewhere. This last animal (deer 1 in table 1) was killed early in the clinical phase of disease for welfare reasons, and this may account for the lower overall magnitude of PrP^d and for the little involvement of forebrain areas. In contrast to the BSE infected deer, the brains of CWD-affected animals showed weak and inconsistent, fine punctate intra-neuronal labelling mainly in neurons of the brainstem and of the deep nuclei of the cerebellum. Like BSE cases, perineuronal labelling was conspicuous in the DMNV; unlike BSE cases, particulate and coalescing PrP^d labelling in the neuropil was prominent, with plaque like accumulations seen throughout most brain areas, often associated with white matter bundles and areas of conspicuous vacuolation (Fig. 3).

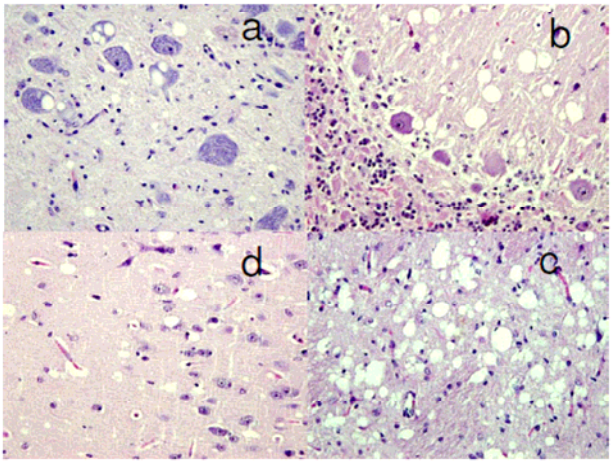

Figure 1
BSE deer. Vacuolation seen in the brainstem (a), the molecular layer of the cerebellum (b), the thalamus (c), and layers V and VI of the cerebral cortex (d).

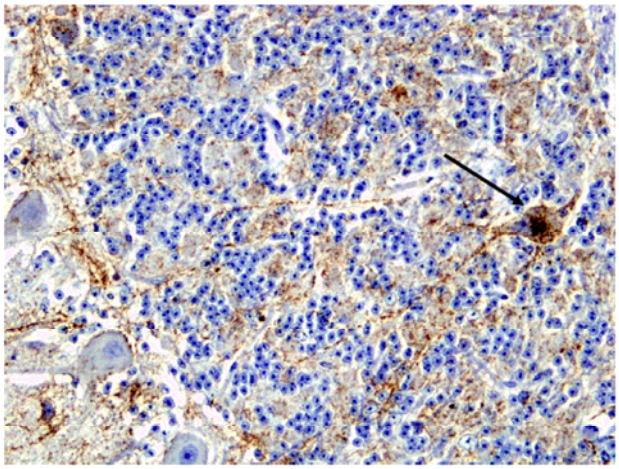

Figure 2
BSE deer. Grey matter PrP^d labelling of neuropil and intense perikaryonal labelling of a Golgi neuron in the granular cell layer of the cerebellum (arrow).

These differences in the patterns of PrP^d accumulation between BSE and CWD resulted in distinct PrP^d profiles that made subjective discrimination relatively straightforward (Figs. 4, 5). Further differences were evident when evaluating the epitope mapping features of the PrP^d molecules of both infections. Samples of obex and midbrain were immunolabelled with N-terminal 12B2 antibody to assess the magnitude of intra-neuronal PrP^d signal in comparison with that obtained with BAR224. In BSE infected deer, the intra-neuronal PrP^d observed in almost all neuronal populations with BAR224 was significantly reduced or lost with 12B2 (Fig. 6), while in CWD affected animals, the lower level of intra-neuronal PrP^d labelled with BAR224 was nevertheless maintained with 12B2 (Fig. 7). No significant difference in extra-cellular signal recovery was seen between CWD and BSE when comparing results with these N-terminal and globular domain antibodies.

Accumulation of PrPd outside the brain

In BSE inoculated deer, PrP^d was detected in all segments of the spinal cord, in autonomic ganglia, cranial and peripheral nerves and in the sensory retina. Strong immunolabelling was found in neurones throughout the enteric nervous system, sometimes in close proximity to nearby lymphoid follicles (Fig 8). However, the Peyer's patches, and all other lymphoid tissues, were negative, as were all other organs examined (for details see [8]). In contrast, CWD infected deer were consistently PrP^d positive in rectal biopsies and in the retropharyngeal lymph node (Fig 9).

Western blotting

The samples from red deer experimentally infected with BSE, either orally or i.c., all gave molecular weight profiles

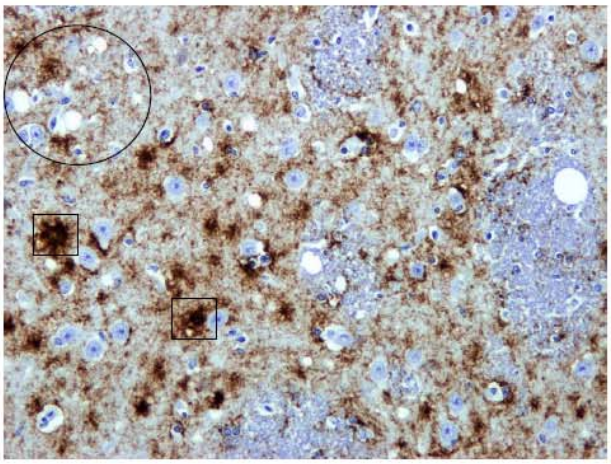

Figure 3
CWD Deer. Vacuolation (oval) and diffuse PrP^d accumulation is present in the grey matter of the striatum of the brain. Focal intense plaque-like accumulations of PrP^d (boxes) are also present.

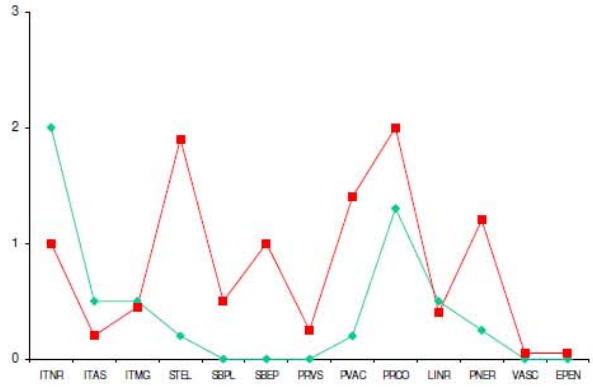

Figure 4
IHC profile of PrP^d types present in BSE infected deer (green) and CWD infected deer (red). Profiles represent a single deer from each of the BSE and CWD infected groups. All six of the deer challenged i.c. with BSE and the single positive orally dosed deer presented consistent profiles on subjective examination that were unlike those of the CWD infected deer. (ITNR, intraneuronal; ITAS, intra-astrocytic; ITMG, intra-microglial; STEL, stellate; SBPL, sub-pial; SBEP, sub-ependymal; PVRS, peri-vascular; PVAC, peri-vacuolar; PRCO, particulate coalescing; LINR, linear; PNER, perineuronal; VASC, vascular; EPEN, Ependymal).

similar to those of cattle BSE, with a lower molecular weight for the unglycosylated protein band compared to that obtained for ovine scrapie, elk CWD or red deer CWD

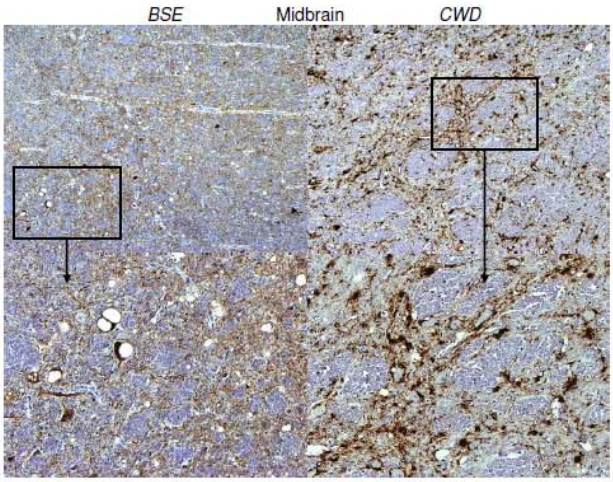

Figure 5
BSE and CWD deer. Low magnification images (top) of midbrain highlight the marked differences seen between the predominantly intra-neronal and difuse particulate labelling of the neuropil that is seen in the BSE infected deer compared to the distinct coalescing plaque-like labelling in the CWD infected deer. The boxes outline the area shown at higher magnification in the lower two images.

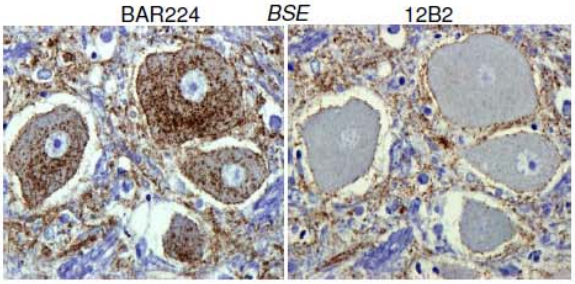

Figure 6
BSE deer. Comparison of intraneuronal labelling with BAR224 (C-terminal) and 12B2 (n-terminal) mAbs showing the significant loss of intraneuronal labelling with 12B2 in BSE infected deer in contrast to CWD infected deer. The extracellular signal was maintained with 12B2 in both the CWD and BSE infected deer.

samples; the latter showed some degree of variability. The antibody affinity was also different between BSE and CWD infected deer. The former showed strong signal with antibodies raised to the C-terminal region of the prion protein (F99) and to globular domain (6H4 and Sha31) and no signal with the antibodies raised to the N-terminus of PrP (P4 and 12B2; Fig. 10), while this reactivity was present in CWD infected animals (Fig. 11).

Discussion

Differences in attack rates and incubation periods observed between oral cattle BSE (16%), intracerebral cattle BSE (100%) and oral deer CWD (100%) included in this study suggest that there is a strong species barrier between cattle and deer in the case of BSE. Although it needs to be pointed out that the remaining five deer orally dosed are still alive and that infection cannot be ruled out,

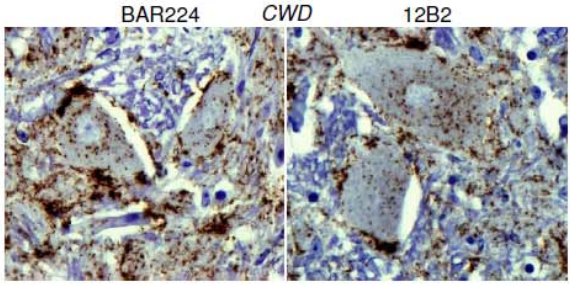

Figure 7
CWD deer. Comparison of intraneuronal labelling with BAR224 (C-terminal) and 12B2 (n-terminal) mAbs showing no reduction of the intracellular signal with the n-terminal targeting antibody.

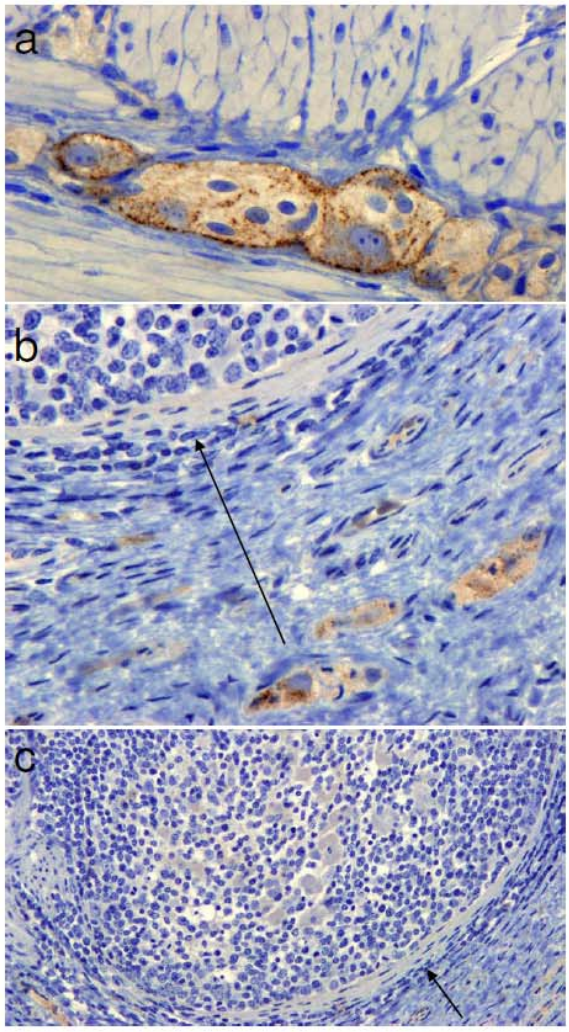

Figure 8
BSE deer. Labelling of PrP^d in cells of the myenteric (a) and sub-mucosal plexi (b) occurred in close proximity to negative Peyer's patches shown at high (b) and low magnification (c). Arrows show the capsule surrounding the same follicle.

this species barrier would agree with the low attack rate found in intracerebral infections of cattle with the CWD agent [24].

The examinations conducted on this experimental material show that infections of red deer with BSE and at least one source of CWD can be clearly differentiated by the same laboratory methods previously used to discriminate between BSE and scrapie in sheep [19,25,26] and goats. Thus, the IHC epitope mapping approach shows that intra-neuronal PrP^d in BSE infected deer is truncated at an

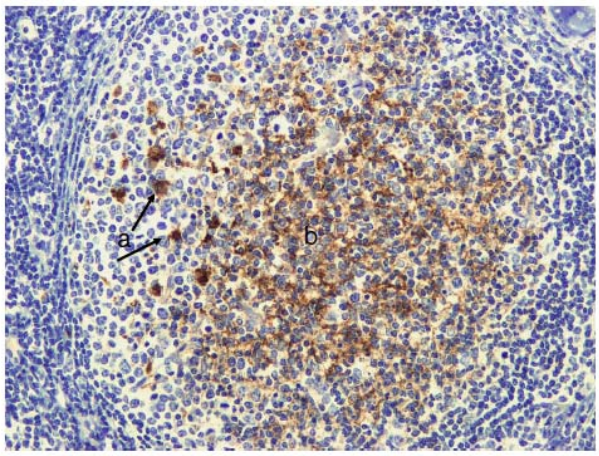

Figure 9
CWD Deer. Labelling of PrP^d in tingible body macrophages (a) and follicular dendritic cells (b) within the secondary follicle of a retropharyngeal lymph node.

epitope further towards the C terminus of the PrP molecule than it does in CWD. This differential truncation also takes place when exogenous enzymes are applied to brain samples as indicated by the discriminatory WB results.

As for the subjectively assessed patterns of IHC PrP^d accumulation -the PrP^d profile-, while these were highly consistent amongst BSE infected deer, some variability was observed in the CWD cases, with one deer showing a slightly divergent profile to the other three and also a considerably higher magnitude PrP^d signal in all brain areas. This variability was also observed in the electrophoretic migration speed of PrP^{res} in the red deer CWD samples, as previously described for elk, mule deer and white tail deer [27], raising the possibility that more than one phenotype of CWD exists even amongst a limited number of animals. Further work is required to investigate whether the IHC profile and molecular weight variation seen in the red deer CWD samples has any significance regarding strain diversity, or is related to other as yet unidentified influences. We were able to examine the obex but not other brain areas of each of the four elk that provided the pooled inoculum for the CWD infected red deer, and the distribution and patterns of PrP^d observed in those samples were indistinguishable from those in the obex of the CWD red deer recipients. Similarly, all four elk used in the inoculum demonstrated almost identical molecular profiles, in contrast with the CWD recipient red deer where some variability was observed. It is thus possible that the host species may have an effect on molecular and pathological phenotypes.

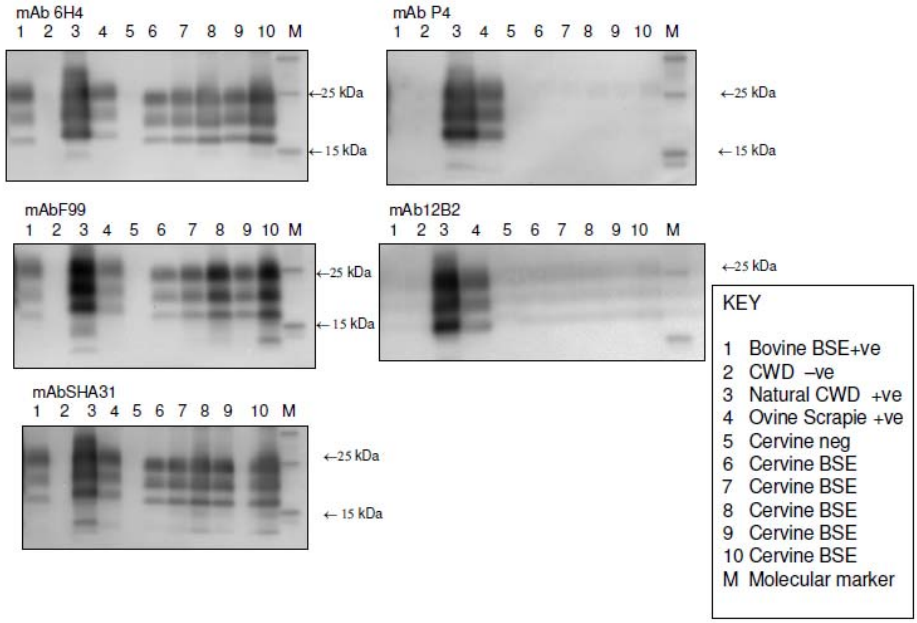

Figure 10
BSE deer. Analysis of brain homogenates by Biorad TeSeE Western Blotting using F99, 6H4, SHA31, P4 and 12B2. Data is not shown for the last of the intracerebrally challenged group or for the single orally challenged BSE positive deer however these presented migration patterns and antibody affinities that were not significantly different from those seen above.

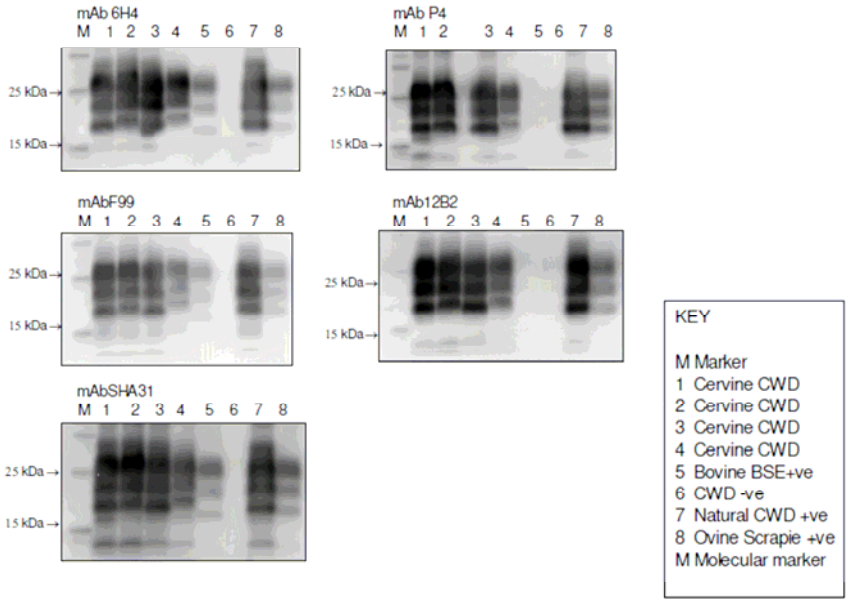

Figure 11
CWD deer. Analysis of brain homogenates by Biorad TeSeE Western Blotting using F99, 6H4, SHA31, P4 and 12B2. The molecular weights of samples from the four red deer experimentally infected with CWD were variable although all of them appeared higher than the cattle BSE control and are more consistent with the naturally infected CWD and scrapie controls.

The minor differences in the topographical distribution of PrP^d found between the BSE orally and i.c. challenged deer may be due to differences in the route of access of infectivity to the brain, as proposed for sheep infected with BSE [12]. In this model, it has been shown that PrP^d accumulates in the porencephalic lesion resulting from the repair process at the site of inoculation, and appears to spread to other neighbouring areas of the cerebral cortex [28]. This pathway would be absent in orally dosed animals, explaining the lesser involvement of rostral areas of the brain.

The fact that the circumventricular organs, which have leaky capillaries and appear to be involved in the neuroinvasion process associated with infectivity present in blood [29], were affected in these BSE infected deer adds support to this notion.

PrP^d was detected in the enteric nervous system and in structures of the peripheral nervous system of the BSE infected deer, regardless of the route of challenge. It is difficult to tell whether that peripheral PrP^d accumulation preceded neuroinvasion or resulted from centrifugal spread, but the lack of LRS involvement in those same deer rather supports the later possibility. This is unlikely to be due to the i.c. route of challenge, as the only orally-dosed deer succumbing to BSE was also negative for PrP^d in lymphoid tissues, and as lymphoid involvement has been shown in BSE i.c. challenged sheep of several *PRNP* genotypes [30]. The absence of LRS involvement seen in BSE-affected red deer is more likely to respond to host genetic factors, as it is reminiscent of cattle BSE, in which the only documented evidence of LRS accumulation of PrPd refers to Peyer's patches after experimental oral challenge [31], and of experimental BSE in sheep bearing the ARR *PRNP* allele [29,30].

The absence of PrP^d in LRS tissues of BSE affected deer, as in natural cattle BSE, might be indicative of a low level of circulating infectivity and suggests that BSE in deer may not be contagious under conditions of natural exposure. Conversely, red deer infected with CWD showed widespread lymphoid involvement, which would be in agreement with the highly contagious nature of this infection in native North American cervids.

Conclusion

European red deer are susceptible to infection with the cattle BSE agent, not only by the intra-cerebral but also by the oral route, and although the clinical signs and spong-

iform change are similar to those of CWD in the same species, these two infections can be easily differentiated. The lack of lymphoid involvement, the PrP^d truncation pattern both "*in vivo*" and "*in vitro*", and the predominantly intracellular accumulation of PrP^d are features of deer BSE that are in contrast with those of deer CWD. However, only one of six deer developed disease after alimentary exposure to 25 g of a BSE brain pool homogenate after an incubation period of nearly 5 years; this suggests a strong species barrier but if a TSE in European red deer should ever be identified then BSE/CWD discrimination would be an urgent priority. To determine whether there are potential naturally occurring BSE-like strains and to determine the degree to which there is strain variation, it would be necessary to examine many more naturally occurring CWD cases. These results will support the ongoing European surveillance for natural TSEs in red deer and the further assessment of potential risk to human health.

Authors' contributions

SM collated the experimental data and wrote the paper. MJ, LG and AB designed the study. SM, SS, LG, MJ and AB examined the tissues. MS, MC and PS performed the biochemical analyses. MD and HR challenged, managed and monitored the deer. All authors have read and approved the final manuscript.

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