

Biochemical and immunohistochemical characterization of feline spongiform encephalopathy in a German captive cheetah

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Feline spongiform encephalopathy (FSE) is a transmissible spongiform encephalopathy that affects domestic cats (*Felis catus*) and captive wild members of the family Felidae. In this report we describe a case of FSE in a captive cheetah from the zoological garden of Nuremberg. The biochemical examination revealed a BSE-like pattern. Disease-associated scrapie prion protein (PrP^{Sc}) was widely distributed in the central and peripheral nervous system, as well as in the lymphoreticular system and in other tissues of the affected animal, as demonstrated by immunohistochemistry and/or immunoblotting. Moreover, we report for the first time the use of the protein misfolding cyclic amplification technique for highly sensitive detection of PrP^{Sc} in the family Felidae. The widespread PrP^{Sc} deposition suggests a simultaneous lymphatic and neural spread of the FSE agent. The detection of PrP^{Sc} in the spleen indicates a potential for prion infectivity of cheetah blood.

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INTRODUCTION

Transmissible spongiform encephalopathies (TSE), or prion diseases, are neurodegenerative diseases affecting humans and numerous mammalian species. Prion diseases include Creutzfeldt–Jakob disease (CJD) in humans, BSE in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk and transmissible mink encephalopathy in farmed mink. Apart from cattle, BSE has also been transmitted by feeding TSE-contaminated feed to exotic ungulates, to domestic cats and to wild cats in zoological gardens.

In domestic cats, feline spongiform encephalopathies (FSE) were first reported in the early 1990s (Wyatt *et al.*, 1990) and 89 such cases have been found in the UK to date (DEFRA, 2009). In contrast, no FSE cases were found by retrospective studies on archived material from 286 felines with neurological disorders that were submitted between 1975 and 1990 (Bradshaw *et al.*, 2004). One FSE case was diagnosed in Norway (Bratberg *et al.*, 1995), one in Liechtenstein (DEFRA, 2009), one in Italy (Zanusso *et al.*, 1998) and two in Switzerland (Demierre *et al.*, 2002, Anonymous, 2005).

FSE cases were also found in 20 captive wild cats: five cheetahs (*Acinonyx jubatus*), five lions (*Panthera leo*), three tigers (*Panthera tigris*), three pumas (*Puma concolor*), three ocelots (*Leopardus pardalis*), one Asian leopard cat (*Prionailurus bengalensis*) and one Asian golden cat (*Catopuma temminckii*) (DEFRA, 2009). Almost all of these FSE cases occurred in animals that were kept in or originated from the UK (Baron *et al.*, 1997). However, there were also two cases reported in wild cats with no direct link to the UK. One was the Asian golden cat which was bred in Germany and exported via the Netherlands to Australia, where it eventually developed FSE (Young & Slocombe, 2003). The second non-UK-correlated FSE case was the offspring of a UK-born and FSE-diseased cheetah in France. The finding of such a case indicates the possibility of vertical/perinatal transmission in cheetahs (Bencsik *et al.*, 2009).

Several sets of data support the hypothesis of there being a common infectious source for FSE and BSE. The diseases were successfully transmitted from three domestic-cat FSE cases to wild-type mice, resulting in incubation periods, clinical signs and histopathological features reminiscent of those observed after cattle-BSE transmissions to these mice (Bruce *et al.*, 1994). The disease-associated scrapie prion protein (PrP^{Sc}) glycotyping patterns (i.e. glycosylation moiety distributions and proteinase cleavage sites) of the FSE-affected cats were similar to those seen in cattle, and

Supplementary tables and a figure are available with the online version of this paper.

were conserved upon transmission to wild-type as well as to transgenic mice (Baron *et al.*, 1999). The regional PrP^{Sc} deposition in the brain of wild-type C57Bl6 or transgenic mice overexpressing ovine PrP [Tg(OvPrP4)] that were challenged with FSE or BSE was also comparable, as shown by paraffin-embedded tissue-blot or immunohistochemical analysis of cortex, hippocampus, hypothalamus and cerebellum (Lezmi *et al.*, 2006; Bencsik *et al.*, 2009). This paper reports the first case of FSE in a captive cheetah detected in Germany. The immunochemical and immunohistochemical examination revealed a BSE-like pattern and a wide distribution of PrP^{Sc} in the central and peripheral nervous system as well as in the lymphoreticular system and other tissues.

RESULTS

A 9-year-old female cheetah from the zoological garden of Nuremberg developed neurological symptoms indicative of FSE in 2007. This animal had been bred and raised in Wassenaar, the Netherlands, for 2 years after its birth. It was first transferred to the zoological garden of Berlin (Tierpark Berlin) and after 4 years brought back to Wassenaar. For the last 2 years of its life the cheetah was kept in the zoological garden of Nuremberg. The neurological signs, which developed over a period of 8 weeks, included ataxia and progressive bilateral hindlimb lameness as well as tremor, dyskinesics and dyspositions of the head. The cheetah was euthanized and necropsied. A wide range of frozen as well as formalin-fixed tissue samples were taken for further studies. FSE was eventually confirmed by the detection of PrP^{Sc} in the CNS using the World Organisation for Animal Health (OIE)-approved scrapie-associated fibrils (SAF) immunoblotting and immunohistochemistry (IHC) techniques.

Immunoblotting

The PrP^{Sc} deposition in the cerebellum, mesencephalon and cerebral cortex of the cheetah was confirmed by

immunoblotting using four different mAb: L42 (Fig. 1a, b), ICSM35 (Fig. 1c, d), 12B2 (Fig. 1e, f) and 3F4 (Supplementary Fig. S1, available in JGV Online). After phosphotungstic acid (PTA) precipitation and proteinase K (PK) digestion, all analysed cheetah samples contained PK-resistant PrP^{Sc} fragments displaying the typical banding pattern representing the diglycosylated, monoglycosylated and unglycosylated moieties. Ovine scrapie and bovine-BSE brain samples served as prion protein (PrP) typing controls. The mAb 3F4 [raised against the MKHM epitope (aa 109–112) of hamster PrP] has been used for feline PrP^{Sc} detection before (Collinge *et al.*, 1996). mAbs L42 (raised against aa 145–163 of hamster PrP) and mAb ICSM35 (detecting an epitope at positions 93–102 of hamster PrP) proved to be similarly suitable. However, mAb 12B2 (which was raised to aa 93–97 of bovine PrP, close to the PK cleavage site) does not retain its reactivity to BSE and BSE-induced FSE-PrP^{Sc} after PK cleavage, whereas scrapie PrP^{Sc} is still detected following such a treatment (Fig. 1f).

Further analysis of cheetah-derived PrP^{Sc} fragments regarding the glycoform ratio and molecular mass were performed using mAb L42 according to the FLI test protocol. The results thereof are summarized in Table 1. The glycoform ratios of PK-digested di-, mono- and unglycosylated PrP^{Sc} fragments revealed differences between the FSE and BSE samples. The glycoform ratios were calculated from five independent immunoblots which were carried out with mAb L42 as detection antibody, and are outlined in Table 1 and displayed in Fig. 3.

Significant differences in the proportions of the diglycosylated form within the overall signal were seen between the BSE and scrapie control (Table 1): the diglycosylated form of the BSE-derived PrP^{Sc} fragment exceeded 60% of the total signal, compared with 46% for scrapie. The proportion determined for the diglycosylated PrP^{Sc} glycoform of the German cheetah H3/07 varied amongst the different brain regions: from 46.28% for the cerebellum, to 53.04% for the mesencephalon and to 55.36% for the cortex. The lowest values, of approximately 44.86% for the diglycosylated fragment, were found in the French FSE sample.

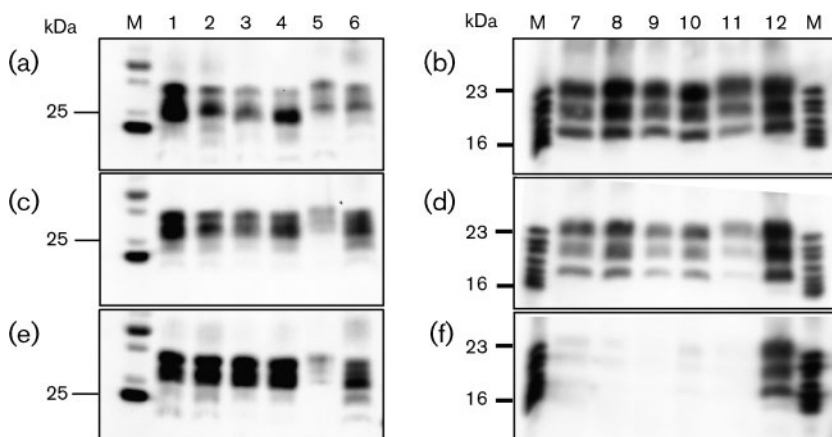


Fig. 1. Western blot analysis of PrP^{Sc} without (a, c and e) or with (b, d and f) PK digestion followed by PTA precipitation. PrP^{Sc} was prepared from a sample from the French cheetah (lanes 1 and 7) and the German cheetah isolate H3/07. PrP^{Sc} of H3/07 derived from cerebellum (lanes 2 and 8), mesencephalon (3 and 9) and cortex (lanes 4 and 10) is shown. PrP^{Sc} control samples originated from the BSE isolate R78/05 (lanes 5 and 11) and the classical scrapie case S3/07 (lanes 6 and 12). Detection was carried out with mAb L42 (a and b), mAb ICSM 35 (c and d) and mAb 12B2 (e and f).

Table 1. Results of the FLI test

Samples were prepared from the French cheetah, the German cheetah (H3/07), cattle BSE ($n=5$) and sheep scrapie ($n=5$).

Sample	PrP ^{Sc} glycoform proportion (%) [*]			MM (kDa) [†]
	2 × glycos	1 × glycos	Unglycos	
Cheetah (France) H3/07	44.86 ± 3.71	31.00 ± 0.82	23.74 ± 3.33	17.94 ± 0.21
Cerebellum	46.28 ± 2.19	36.80 ± 2.38	16.94 ± 2.83	17.82 ± 0.74
Mesencephalon	53.04 ± 0.42	29.22 ± 0.67	17.70 ± 0.80	17.77 ± 0.72
Cortex	55.36 ± 1.69	27.48 ± 0.94	17.16 ± 1.08	17.56 ± 0.63
BSE	65.54 ± 1.84	25.40 ± 1.86	9.06 ± 0.82	17.95 ± 0.31
Scrapie	46.56 ± 1.61	33.96 ± 2.03	19.50 ± 1.79	18.46 ± 0.11

^{*}Percentages are indicated as means of the diglycosylated (2 × glycos), monoglycosylated (1 × glycos) and unglycosylated (unglycos) PrP^{Sc} bands. Data are indicated as arithmetic means ± SD of five values.

[†]Molecular mass of the unglycosylated PrP^{Sc} band.

The proportion of the monoglycosylated PrP^{Sc} of H3/07 also showed a different signature in the different brain regions (Table 1). The cerebellum sample exhibited a high proportion of approximately 36.8%, in contrast to 29% for the mesencephalon and 27.48% for the cortex. Marginal differences were seen in the proportion of the unglycosylated fragment. With regard to the monoglycosylated fragment, the French cheetah harboured the highest ratio of about 23.74%. The distribution of the glycoprofile is summarized in a triangular plot (Fig. 2). The values determined for the cheetah represent an intermediate glycoprofile between the BSE and scrapie profiles.

The molecular mass of the unglycosylated PrP^{Sc} fragment is an additional feature for discrimination between scrapie and BSE (Table 1), and was calculated from five independent immunoblots incubated with mAb L42. The

unglycosylated BSE fragment displayed a molecular mass which was about 0.5 kDa lower than that of the scrapie-derived fragment. The cheetah-derived PrP^{Sc} fragments displayed a molecular mass similar to that of the BSE

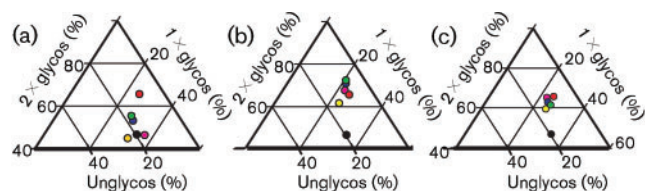


Fig. 2. Triblot diagram derived from glycosylation patterns of FSE samples and PMCA-derived PrP^{res} fragments. (a) Glycoprofile of FSE samples: cortex (green), mesencephalon (blue), cerebellum (magenta), French FSE (yellow) as compared with a classical BSE (red) and scrapie controls (black). (b) Glycoprofile of PrP^{res} fragments after the first round of PMCA: cortex (green), mesencephalon (blue), cerebellum (magenta), French FSE (yellow) compared with unamplified classical BSE (red) and scrapie controls (black). (c) Glycoprofile of PrP^{res} fragments after the second round of PMCA: cortex (green), mesencephalon (blue), cerebellum (magenta), French FSE (yellow) compared with unamplified classical BSE (red) and scrapie controls (black).

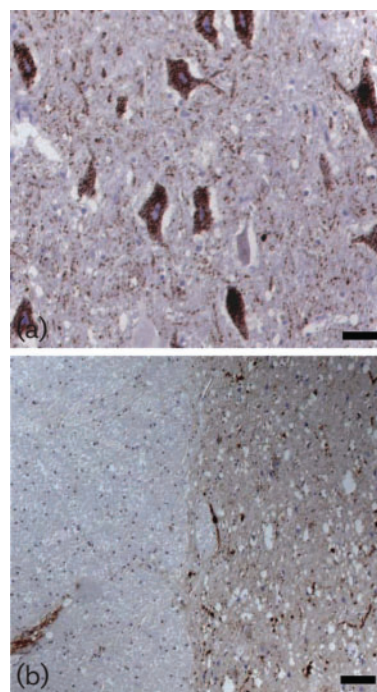


Fig. 3. PrP^{Sc} accumulation in the brain of a cheetah, IHC with mAb 2G11 and Nomarski interference. (a) Brainstem/n. hypoglossus, severe intraneuronal and moderate diffuse fine to coarse granular immunolabelling. (b) Striatum/nc. lentiformis, grey matter with severe, thread-like linear and moderate diffuse fine granular immunolabelling, white matter with no staining reaction. Bars, 50 μ m.

sample, which were significantly lower than that determined for the scrapie sample.

Histopathology and IHC on CNS samples

Pronounced spongiform alterations were seen in the midbrain, thalamus and hypothalamus as well as in the striatum [nucleus (nc). caudatus, nc. lentiformis], while the brainstem at the level of the obex (especially nc. tractus solitarius, nc. spinalis nervus (n.) trigemini) and the cerebellum (all layers) were altered to a lesser degree. The vacuolation was largely confined to the neuropil and rarely seen in neurons. Moreover, a moderate gliosis accompanied by microglial nodules, as well as the necrosis of neurons, was visible.

Both antibodies used in the immunohistochemical examination revealed a clear accumulation of PrP^{Sc} in all regions analysed. PrP^{Sc} depositions were most prominent in the grey matter of the brainstem, midbrain, thalamus and cerebellum. In the cerebellum, the molecular and the granular layer as well as the cerebellar nuclei were equally involved, while Purkinje cells were negative. In the cerebral cortex, PrP^{Sc} depositions were found primarily in the internal pyramidal layers IV–VI, while the white matter stained only weakly, and no subpial reaction pattern was detectable. Regarding the cellular level, only a few reaction patterns can be seen. Glial labelling was visible throughout the brain. Intra-neuronal staining reaction was most prominent in the brainstem (Fig. 3a), cerebellar nuclei, substantia nigra, nc. ruber and thalamus. At the dorsal motor nucleus of the n. vagus, an additional perineuronal reaction pattern was seen. A mild linear PrP^{Sc} reaction pattern was observed throughout the brain, while in the nc. lentiformis and the substantia nigra a thread-like appearance was striking (Fig. 3b). A diffuse fine-granular labelling of the neuropil was seen throughout the brain. A coarse-to-coalescing deposition was confined to the brainstem, midbrain and thalamus.

In the spinal cord, mild spongiform alterations of the neuropil, mild gliosis and neuronal necrosis were seen in all regions of the grey matter. Randomly scattered signs of degeneration were observed in the white matter. By IHC, PrP^{Sc} was detected mainly in the grey but also in the white matter of all spinal-cord regions in form of: (a) severe diffuse fine to coarse granular and (b) moderate to severe intracytoplasmic staining reactions of neurons and in glial cells. Moreover, mild linear reaction patterns in the neuropil, as well as mild intraependymal and perivascular staining reactions, were seen. The PrP^{Sc} accumulation in the white matter was confined mainly to septum areas and ventral-horn-derived axons.

Peripheral PrP^{Sc} distribution

A wide range of peripheral tissue samples of this animal were examined using IHC (Table 2). Mild axonal depositions of PrP^{Sc} were detectable throughout the peripheral nervous system, in spinal ganglia, in nerves of

the tongue (Fig. 4a) and in the sciatic nerve. Furthermore, an accumulation of PrP^{Sc} was also seen in glial cells of the optic nerve (Fig. 4b), as well as in neurons and satellite cells of the plexus pelvius. In the enteric nervous system, mostly in the myenteric but also the submucosal parts, both neurons and satellite cells were affected.

Additionally, moderate to severe intra- and perineuronal immunolabelling of PrP^{Sc} was seen in the sympathetic neurons of the adrenal-gland medulla (Fig. 4c). Multifocally, the chromaffin cells revealed a mild pericellular staining reaction too. However, the cortex of the adrenal gland showed the most prominent accumulation of PrP^{Sc} outside the CNS, with a severe intra- and pericellular immunoreaction confined to the zona fasciculata (Fig. 4d). Most interestingly, neither morphological alterations nor deviations of the associated hormone levels in the blood were detectable (data not shown).

In the lymphoreticular system PrP^{Sc} was visible in the spleen and the mandibular lymph node. In the spleen only a few depleted follicles were detectable and most of them showed a clear granular intracytoplasmic PrP^{Sc} accumulation in the small peripheral mononuclear cells (Fig. 4e). The PrP^{Sc} finding for spleen was confirmed by protein misfolding cyclic amplification (PMCA; see below). Monocytes and a fine dendritic network were stained in the light central zone of the lymph node follicles (Fig. 4f). An additional staining was performed by using antibody BG4, which binds the N terminus of PrP^{Sc} and which is known to detect follicular dendritic cell (FDC)-associated PrP^{Sc} only (Fig. 4g).

While most parenchymas that were examined by IHC (Table 2) showed no detectable accumulation of PrP^{Sc}, the kidney revealed an ambiguous staining reaction of individual glomeruli that was interpreted as being artificial. To further highlight this finding, kidney samples were subjected to PMCA and to mouse challenge studies. Using PMCA, PrP^{Sc} from a kidney of H3/07 could not be clearly determined (see below). However, challenge studies using ovine transgenic mice (Tgshp XI), that were inoculated intracerebrally with kidney samples, revealed unambiguous proof of the presence of infectivity. Accumulated PrP^{Sc} could be detected in the brains of six out of eight mice used for the bioassay, with a mean incubation period of 300 days (Fig. 6b; Supplementary Table S2, available in JGV Online). A simultaneous challenge experiment using brainstem samples from H3/07 revealed a transmission rate of 100% (9/9), and an incubation period of 244 days (Supplementary Table S2).

PMCA

In order to investigate FSE propagation *in vitro*, samples of cheetah-derived FSE were subjected to a PMCA analysis. For amplification of both BSE and FSE PrP^{Sc} material, we used a 10% brain homogenate of Tgbov XV mice as the substrate. Due to the eightfold overexpression of bovine

Table 2. Results of the immunohistochemical analysis for PrP^{Sc} in the spinal cord, peripheral nervous system, lymphoreticular system and different parenchyma

Extent of immunolabelling: --, negative; +, mild; ++, moderate; + + +, severe.

Tissue sample	PrP ^{Sc} IHC (mAb L42 and 2G11)	Positive structure
Spinal cord		
Cervical	+++	Grey (intracellular, cell surface, ependyma) and white matter (septal area, axons)
Thoracic		
Lumbar		
Peripheral nervous system		
N. opticus	+	Astro-/oligodendrocytes
N. ischiaticus	+	Axons
Plexus pelvinus (urethra)	+	Neurons, satellite cells
Lymphoreticular system		
Spleen	+	Follicles: single small, peripherally located, mononuclear cells
Lnn. mandibularis	++	Follicles: monocytes, follicular dendritic cells
Parenchyma		
Lung	--	--
Heart	--	--
Liver	--	--
Gut (small intestine)	+	Enteric nervous system (myenteric and submucosal plexus)
Kidney	Inconclusive	Glomerula: highly ambiguous staining reaction
Adrenal gland	+++	Cortex (zona fasciculata) and medulla (mainly neurons)
Bladder	--	--
Ovary	--	--
Uterus	--	--
Muscle		
Tongue	+	Axons (peripheral nerves)
Diaphragm	--	--
Hindlimb muscle	--	--

cellular prion protein (PrP^C) in these transgenic mice, this substrate yields a higher PMCA amplification rate than normal bovine brain (data not shown). FSE and BSE brain samples were mixed with Tgbov XV brain homogenate at a 1:1000 dilution. After 144 cycles of PMCA, samples were diluted 1:10 in Tgbov substrate and subjected to a second round of PMCA, also of 144 cycles. All samples were digested with PK, then PTA precipitated and immunoblotted (Fig. 5). Compared with the unamplified controls (Fig. 5a, lanes 4, 7, 10, 13 and 16), a significant increase in signal for PK-resistant PrP^{res} fragments was detected after the first round of PMCA for FSE, including cerebellum (Fig. 5a, lane 5), mesencephalon (Fig. 5a, lane 8), cortex (Fig. 5a, lane 11), the French cheetah (Fig. 5a, lane 14) and BSE (Fig. 5a, lane 17). After a 1:10 dilution in Tgbov substrate and a second round of PMCA, PrP^{res} fragments were generated at the same level as the first round (Fig. 5a, lanes 6, 9, 12 and 15), which indicates autocatalytic replication of PrP^{res}. Analysis of PMCA generated PrP^{res} fragments revealed a unique glycoform profile for the first (Fig. 2b) and second (Fig. 2c) PMCA rounds. In contrast to the original FSE-derived PrP^{Sc} glycosylation pattern

(Table 1), the diglycosylated form of PrP^{res} exceeded 60% and >70% in the case of mesencephalon and cortex (Fig. 2b; Supplementary Table S1, available in JGV Online). In the second round, the values of the diglycosylated form stabilized at a mean level of approximately 60% (Fig. 2c; Supplementary Table S1). No PrP^{res} fragments originated from the brain of an uninfected cat (Fig. 5a, lanes 2–3) nor from Tgbov substrate alone (Fig. 5b, lanes 2–3). As an additional control, we analysed the influence of the PMCA substrate (Fig. 5b, lanes 4–8). Starting from a 1:10 dilution of the PrP^{Sc} seed (Fig. 5b, lane 4), sonication in Tgbov substrate induced PrP^{res} formation (Fig. 5b, lanes 7–8). In contrast to this, for PrP^{Sc} seeds devoid of Tgbov substrate the PrP^{res} level decreased below the detection limit (Fig. 5b, lanes 5–6). This indicates that PMCA is a template-assisted process and that simple sonication of PrP^{Sc} aggregates does not release additional epitopes nor does it increase staining intensity.

To confirm the PrP^{Sc} deposition in the spleen of the cheetah, spleen samples were analysed by PMCA. Prior to PMCA, a PTA precipitation was carried out to remove

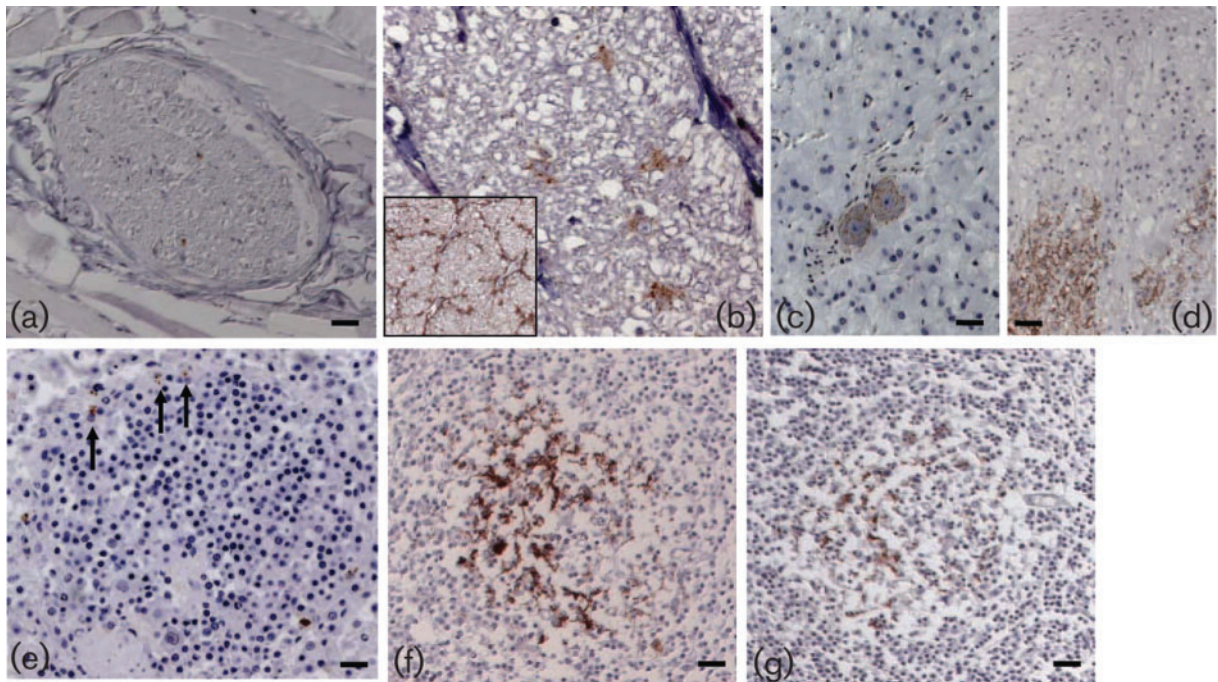


Fig. 4. PrP^{Sc} accumulation in different tissues of a cheetah, IHC with Nomarski interference. (a) Peripheral nerve in the tongue with a mild axonal staining reaction (mAb 2G11). (b) Optic nerve among others with glial immunolabelling (mAb 6C2). Inset showing the same region with a clear GFAP staining reaction (pAb N1506 RTU) indicative of glial cells. (c, d) Adrenal gland with intra- and perineuronal staining reaction (mAb 2G11) in the medulla (c) and intra- and pericellular immunolabelling in the zona fasciculata (d). (e) Depleted follicle of the spleen with a fine granular intracellular reaction pattern (mAb 2G11) related to small peripheral mononuclear cells (arrows). (f) Peripheral lymph node showing a globular as well as a net-like reaction pattern (mAb 6C2) within a follicle. (g) Adjacent section of the peripheral lymph node shown in (f) but stained with antibody BG4 (N-terminal), showing a weak net-like immunolabelling, indicating the dendritic network in the same follicle. Bars, 20 μm (a–d, f, g); 10 μm (e).

PMCA-inhibitory blood contaminants and also to increase the PrP^{Sc} concentration. Sample pellets were then mixed with the Tgbov XV substrate and subjected to PMCA. After 144 cycles a significant number of PrP^{res} fragments were detected in the spleen sample of H3/07 (Fig. 6, lane 4), in contrast to the unamplified control sample (Fig. 6, lane 2). The spleen of an uninfected domestic cat (*Felis catus*) was used as a negative control. No PrP^{res} fragments were detected before (Fig. 6, lane 1) or after (Fig. 6, lane 3) the amplification procedure. As a positive control, samples of the cortex of H3/07 were PTA precipitated and amplified by PMCA (Fig. 6, lane 5). In addition, a PK-digested BSE control from a confirmed BSE case (R78/05) was used (Fig. 6, lane 6). The same experiments were done with kidney samples from H3/07. In summary, we could detect PrP^{res} in the spleen sample of H3/07 in four independent PMCA reactions. In 2/4 cases PrP^{res} was detected in the first round, and in all cases PrP^{res} was detected in the second round. In the case of kidney from H3/07, we only detected PrP^{res} once in the PMCA reaction. However, repeated PMCA reactions could not confirm this finding. We concluded from this investigation that PrP^{res} detection from spleen could not be confirmed with certainty by PMCA.

DISCUSSION

We report here the third FSE case in a wild cat (the second case in a cheetah) that is not directly linked to an infection in the UK, but rather to an infectious source in the Netherlands or Germany. This animal succumbed to disease, eventually, in a zoological garden in Germany. The first non-UK FSE case in a cheetah was diagnosed in France. However, that animal was the offspring of an FSE-infected cheetah from the UK and had thus most probably been infected pre- or perinatally. It remains unclear where the here-described cheetah (H3/07) contracted the FSE infection. It was born in the Netherlands and lived there for periods totalling almost half of its life. During the rest of its life it was exhibited in zoological gardens in Germany. Interestingly, this animal was born in the same breeding station as an Asian golden cat that was later exported to Australia, where it developed FSE (Young & Slocombe 2003).

Earlier histopathological, immunohistochemical, immunohistochemical and mouse transmission studies have linked FSE cases in domestic and wild cats with BSE in cattle, as far as possible (Wyatt *et al.*, 1991; Bruce *et al.*, 1994; Baron *et al.*,

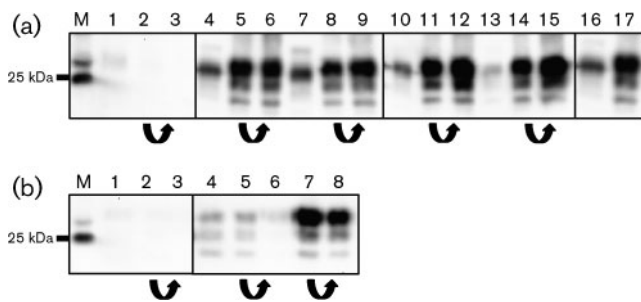


Fig. 5. Western blot of an *in vitro* amplification of BSE and FSE samples using PMCA. (a) Negative feline brain before (lane 1) and after (lanes 2–3) PMCA procedure. FSE samples include cerebellum (lanes 3–6), mesencephalon (lanes 7–9), cortex (lanes 10–12) and the French FSE case (lanes 13–15), as well as a BSE sample (lanes 16–17). Corresponding samples were seeded at a 1 : 1000 dilution (lanes 1, 4, 7, 10, 13 and 16). After 144 cycles of PMCA a significant increase in PK-resistant PrP^{res} fragments was detected for FSE and BSE samples (lanes 5, 8, 11, 14 and 17). PrP^{res} fragments were diluted 1 : 10 and again subjected to PMCA, consisting of 144 cycles (lanes 6, 9, 12 and 15). (b) Negative control (Tgbov substrate) before (lane 1) and after (lanes 2–3) PMCA. H3/07-derived PrP^{Sc} from cortex in a 1 : 10 dilution (lane 4), after two consecutive rounds of PMCA lacking Tgbov substrate (lanes 5–6) or in combination with Tgbov substrate (lanes 7–8). All samples were digested with PK and were PTA precipitated. Detection was carried out with mAb L42. Arrows indicate the 1 : 10 dilution of PrP^{res} substrate and the subsequent round of PMCA.

1999; Lezmi *et al.*, 2003, 2006; Young & Slocombe 2003; Bencsik *et al.*, 2009). The data for the here-reported cheetah case are in line with these earlier findings. Although the Tgshp XI model has never been validated systematically as a strain typing assay, it should be mentioned that the observed neuropil vacuolation and the limited IHC reaction pattern display more similarities to BSE than to scrapie.

PrP^{Sc} glycotyping patterns were generally BSE-like, though the PrP^{Sc} deposition in the cerebellum displayed a scrapie-like pattern instead. This deviation may be a consequence of the local preference of certain PrP^C glycosylation forms, which have also been found in mouse (Beringue *et al.*, 2003) and human (Kuczius *et al.*, 2007) brains. A mixed scrapie- and BSE-like phenotype has also been described for the scrapie strain CH1641 (Stack *et al.*, 2002; Baron *et al.*, 2004). However, the additional biochemical parameters (antibody binding and molecular mass of the unglycosylated PrP^{Sc} fragments) indicate unambiguously the BSE-like properties of the FSE samples. In particular, the absence of the staining reaction with mAb 12B2 seen in the French and German FSE cheetah samples is indicative of a common BSE-like origin.

The unique properties of the FSE samples were found by challenge experiments with ovine transgenic mice (Tgshp

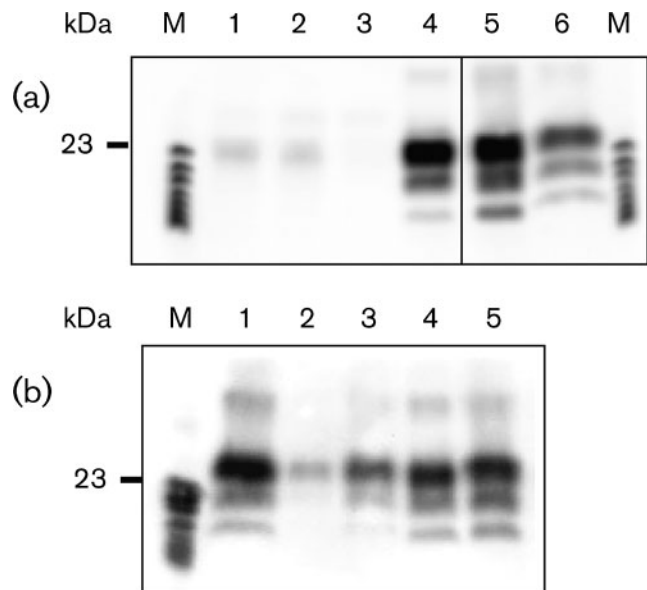


Fig. 6. (a) Western blot of an *in vitro* amplification of spleen samples from an FSE-infected cheetah after PK digestion and PTA precipitation. Control samples of a negative feline spleen (lane 1) and spleen of H3/07 (lane 2) were amplified using PMCA. After 144 cycles no signal was seen in the feline spleen control (lane 3) but PrP^{res} could be confirmed in the spleen of H3/07 (lane 4). PTA-precipitated samples from the cortex of H3/07 (lane 5) and a BSE sample (lane 6) served as positive controls. Detection was carried out with mAb L42. (b) Western blot analysis of PrP^{Sc} isolated from the brain of Tgshp XI mice challenged with kidney samples from the cheetah H3/07 (lanes 1–4). A BSE cattle brain served as a positive control (lane 5). Detection was carried out with mAb L42.

XI). In previous challenge experiments using Tgshp XI mice (Kupfer *et al.*, 2007) the mice exhibited a high susceptibility to sheep scrapie brain (incubation time, 244 days) as compared with a reduced susceptibility to cattle BSE brain (incubation time, 305 days). Here, the mice displayed intermediate susceptibility to FSE (incubation time, 244 days), which can be explained by the occurrence of strain-specific interactions of PrP^{Sc} with the PrP of the transgenic mice.

Previous investigations of the peripheral PrP^{Sc} distribution in wild cats were frequently hampered by the poor quality of the samples available (Ryder *et al.*, 2001; Lezmi *et al.*, 2003). Our results now confirm that PrP^{Sc} can be found in all parts of the peripheral nervous system. Similar to BSE in cattle where the spread to the spinal cord follows largely efferent nerve fibres (Hoffmann *et al.*, 2007), the preferential PrP^{Sc} deposition in the adjacent ventrolateral white matter of the spinal cord of the cheetah is indicative of a similar pathway. However, it is also interesting to note that axonal PrP^{Sc} depositions were only seen in motor nerves (tongue, sciatic nerve) but not in the autonomic nervous system (plexus pelvinus), suggesting differences in

the spread of PrP^{Sc} between the various parts of the nervous system. The intraneuronal detection of PrP^{Sc} in the medulla of the adrenal gland may be associated with the spread within the autonomous nervous system, as described for scrapie in sheep (Jeffrey *et al.*, 2001). However, the highest accumulation of PrP^{Sc} outside the CNS was found in the zona fasciculata of the adrenal gland, which is in accordance with a previous report about a cheetah but in contrast to the reaction pattern seen for sheep scrapie (Jeffrey *et al.* 2001; Lezmi *et al.*, 2003). PrP^{Sc} was also found in the lymphoreticular system as described previously (Ryder *et al.*, 2001; Lezmi *et al.*, 2003). In the lymph nodes examined, FDCs and monocytic cells were affected, whereas [in contrast to the report by Ryder *et al.* (2001)] PrP^{Sc} depositions in the spleen were found in B-cell-like cells surrounding depleted follicles. Whether this is an indication of an intrinsic haematogenous spread remains to be determined.

Furthermore, mouse challenge experiments documented the presence of prion infectivity in kidney samples. However, as shown by others (Ryder *et al.*, 2001; Lezmi *et al.*, 2003), the staining reaction seen in the glomeruli with different antibodies by IHC was interpreted as artificial, being similar to a labelling described by others for renal tubules. A reaction pattern involving epithelial cells or the interstitium of the renal papillae was not detectable (Ligios *et al.*, 2007; Siso *et al.*, 2008). Therefore, it remains unresolved which structures in the kidney contain the infectivity.

Apart from these conventional PrP^{Sc}-detection techniques, we were able to demonstrate the amplification of PrP^{Sc} in the brain and spleen of this cheetah by PMCA. By the use of two consecutive rounds of PMCA, we could demonstrate the autocatalytic propagation of feline PrP^{res} fragments in bovine PrP^C substrates from transgenic Tgbov XV mice. PMCA was used recently to amplify PrP^{Sc} from hamster and mouse scrapie (Saborio *et al.*, 2001; Castilla *et al.*, 2008), CWD (Kurt *et al.*, 2007), CJD (Jones *et al.*, 2007), ovine scrapie (Thorne & Terry, 2008) and BSE in cattle (Richt *et al.*, 2007). The PMCA-amplified PrP^{res} resembled bovine PrP^{Sc} biochemically in regard to its PK cleavage site and glycosylation pattern. These data provide further evidence of the close relationship between both strains and of a potential common origin.

METHODS

Animal samples. At necropsy a large collection of frozen and formalin-fixed tissue samples were obtained from the German FSE-diseased cheetah (H3/07). Frozen samples from an FSE-diseased cheetah from France were kindly provided by T. Baron, Agence Française de Sécurité Sanitaire des Aliments-Lyon, Unité ATNC, Lyon, France. Other controls included a classical sheep scrapie case (S3/07) and a classical BSE case (R78/05), from Germany.

OIE immunoblotting. The deposition of PrP^{Sc} in the brainstem at the level of the obex region of the cheetah was verified by the OIE-approved SAF immunoblotting technique using the PrP-specific

monoclonal detection mAb L42 (Harmeyer *et al.*, 1998; Anonymous, 2008).

Histopathology and IHC. For histopathological studies, tissue samples were fixed in 4% neutral-buffered formalin and treated for 1 h with 98% formic acid, rinsed in tap water, embedded in paraffin and cut into 3 µm sections, and stained with haematoxylin and eosin. For IHC a Dako EnVision kit and the primary mAbs 2G11, L42, 6C2 and BG4 were used for PrP. Glial fibrillary acidic protein (GFAP) was stained by the avidin-biotin-complex (ABC) method using a polyclonal antibody (pAb) (N1506 RTU; DAKO). Sections were mounted on SuperFrost Plus slides (Menzel-Gläser) and treated with 98% formic acid for 15 min for mAb 2G11, mAb L42 and pAb GFAP, or for 30 min for mAbs 6C2 and BG4. This was followed by a 5 min rinse in tap water and then by incubation with 3% H₂O₂ (Merck) in methanol for 30 min. Depending on the primary antibody applied, there followed a digestion with 4 µg PK ml⁻¹ (Boehringer Mannheim) at 37 °C for 15 min in the case of mAb L42 and 5 min in the case of GFAP. Alternatively, an autoclaving for 30 min in citrate buffer (pH 6.0) was performed in the case of mAbs 2G11, 6C2 and BG4. mAb L42 (Harmeyer *et al.*, 1998) was applied at a dilution of 1:250, mAb 2G11 (Andréoletti *et al.*, 2000) at a dilution of 1:350 (1.43 µg ml⁻¹), mAb 6C2 (Jan Langeveld, Central Veterinary Institute, Lelystad, The Netherlands) at a dilution of 1:50, mAb BG4 (TSE Research Centre) at a dilution of 1:150 and pAb GFAP at a dilution of 1:1500, in goat serum for 2 h. Negative controls were treated with goat serum alone. Secondary antibody coupled to horseradish peroxidase (Mouse EnVision HRP; Dako) was used to detect mAbs. For the pAb, immunodetection was amplified using Vector ABC-elite avidin-horseradish peroxidase-biotin complex (Vector Laboratories). Slides were developed using diaminobenzidine and counterstained with Mayer's haematoxylin before examination by light microscopy.

SDS-PAGE and immunoblotting. Samples were loaded onto SDS-polyacrylamide gels containing 16% bisacrylamide. After electrophoresis, proteins were transferred to a PVDF membrane in a semi-dry chamber. Membranes were incubated in blocking buffer [PBS (pH 7.2) containing 0.1% Tween 20 and 5% non-fat dried milk powder], followed by incubation for 60 min with the corresponding antibody. Membranes were washed three times for 10 min each with PBS containing 0.1% Tween 20 and incubated for 60 min with a secondary antibody conjugated to alkaline phosphatase (AP) (goat anti-mouse-AP). Membranes were washed again, three times for 10 min each with PBS containing 0.1% Tween 20. Subsequently, the chemiluminescence substrate CDP-Star (Tropix) was applied and incubated on the membrane for 5 min. Visualization was achieved using a VersaDoc imaging system (Bio-Rad).

Discriminatory FLI test (PTA immunoblotting). Cheetah samples were analysed by using a TSE discriminatory test (FLI-test) which includes a PTA precipitation of PrP^{Sc} followed by SDS-PAGE and immunoblotting as described by Gretzschel *et al.* (2005). Four different mAb were used: L42 (R-Biopharm), 3F4 (Covance), 12B2 (Signet Laboratories) and ICSM35 (D-Gen). Banding patterns were revealed using the chemiluminescence substrate CDP-Star (Tropix). After incubation for 5 min, emitted light signals were recorded by a photo imager system (VersaDoc; Bio-Rad).

PMCA. PMCA was generally carried out following published protocols (Castilla *et al.*, 2005a, b, 2006). Frozen brain and tissue samples were homogenized at 10% (w/v) in PMCA conversion buffer [PBS containing 150 mM NaCl, 1.0% Triton X-100 and 1 × complete protease inhibitor cocktail (Roche)] and stored at -20 °C. Tgbov XV mouse brains from Tgbov XV mice, which overexpress bovine PrP (Buschmann & Groschup, 2005), were used as the PrP^C substrate. Mouse brains were perfused with PBS and were homogenized at 10%

(w/v) in PMCA conversion buffer. All homogenates were spun down at 1500 g for 30 s and supernatants were immediately frozen at -70°C . Analyte samples were diluted 1:10 in Tgbov XV brain substrates (total volume, 100 μl) and transferred to 0.2 μl PCR tubes.

Spleen and kidney samples were subjected to a modified PTA precipitation prior to cyclic amplification. Five hundred microlitres of homogenate [10% (w/v) in PMCA conversion buffer] was incubated with 500 μl PBS (pH 7.2) containing 4% (w/v) sarkosyl for 30 min at 37°C followed by incubation with PTA (0.3% w/v). After centrifugation, pellets were dissolved in Tgbov XV brain homogenate, sonicated for 60 s, apportioned into 0.2 ml PCR tubes and subjected to PMCA.

The tubes were then placed into a thin-walled adaptor and put on a microplate module of a horn sonicator (model 3000; Misonix). Samples were exposed to 144 cycles, each cycle consisting of a sonication step of 20 s at a potency of 190–200 W (level 7) and an incubation step of 30 min at 35°C . After the first round of 144 cycles, samples were diluted 1:10 in Tgbov substrate and subjected to a further round, also consisting of 144 cycles.

PMCA products were then incubated with PK (final concentration of $75\ \mu\text{g ml}^{-1}$) for 60 min at 55°C . In contrast to the original PrP^{Sc} fragments, amplified PK-resistant PrP fragments were designated PrP^{res}. After stopping the reaction with 0.5 mM PMSF, the samples were subjected to a PTA precipitation (see above). The pelleted samples were resuspended in 50 μl loading buffer [pH 6.8, containing 0.1 g SDS ml^{-1} , 25 mM Tris/HCl (pH 7.4), 0.5% β -mercapto-ethanol and 0.001% bromophenol blue], denatured at 95°C for 5 min and analysed by SDS-PAGE and immunoblotting.

Transgenic mouse challenge studies. Challenge studies were performed to analyse the susceptibility of Tgshp XI mice, which overexpress full-length ovine PrP four- to eightfold (Kupfer *et al.*, 2007). Groups of 12–15 mice were intracerebrally inoculated with 30 μl of 10% tissue homogenates from brainstem and kidney diluted in sterile 0.9% sodium chloride. All mice were assessed for the onset of clinical symptoms at least twice weekly. Mice showing clinical signs were sacrificed and their brains tested for the accumulation of PrP^{Sc} by a PTA immunoblotting method, as described before.

Incubation times were calculated as the time between inoculation and death. All mice in an experiment were tested for PrP^{Sc} accumulation in their brains and only those with positive results were included in the calculation of incubation times.

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