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Absence of classical and atypical (H- and L-) BSE infectivity in the blood of bovines in the clinical end stage of disease as confirmed by intraspecies blood transfusion

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Abstract

While the presence of bovine spongiform encephalopathy (BSE) infectivity in the blood of clinically affected sheep has been proven by intraspecies blood-transfusion experiments, this question has remained open in the case of BSE-affected cattle. Although the absence of infectivity can be anticipated from the restriction of the agent to neuronal tissues in this species, evidence for this was still lacking. This particularly concerns the production and use of medicinal products and other applications containing bovine blood or preparations thereof. We therefore performed a blood-transfusion experiment from cattle in the clinical end stage of disease after experimental challenge with either classical (C-BSE) or atypical (H- and L-) BSE into calves at 4–6 months of age. The animals were kept in a free-ranging group for 10 years. Starting from 24 months post-transfusion, a thorough clinical examination was performed every 6 weeks in order to detect early symptoms of a BSE infection. Throughout the experiment, the clinical picture of all animals gave no indication of a BSE infection. Upon necropsy, the brainstem samples were analysed by BSE rapid test as well as by the highly sensitive Protein Misfolding Cyclic Amplification (PMCA), all with negative results. These results add resilient data to confirm the absence of BSE infectivity in the donor blood collected from C-, H- and L-BSE-affected cattle even in the final clinical phase of the disease. This finding has important implications for the risk assessment of bovine blood and blood products in the production of medicinal products and other preparations.

INTRODUCTION

Bovine spongiform encephalopathy (BSE), together with scrapie in sheep and goats, chronic wasting disease (CWD) in cervids and Creutzfeldt–Jakob disease (CJD) in humans, belongs to the group of transmissible spongiform encephalopathies (TSE). TSE pathogenesis studies in different host species have revealed some major differences in the TSE pathogenesis observed in different species, even for the same TSE strain, such as for example bovine and ovine BSE [1–5]. While in bovine BSE, depositions of the pathological and disease-related prion protein PrP^{Sc} as well as infectivity is generally restricted to the central and peripheral nervous system until the final stage of disease [2, 3, 6–11], PrP^{Sc} as well as infectivity are easily detectable in the peripheral nervous and lymphoreticular systems throughout the incubation period in BSE or scrapie-infected sheep [1, 4, 12–14]. For atypical BSE, the presence of PrP^{Sc} depositions in peripheral nervous and muscular tissues have been described [15–18], albeit at distinctly lower levels as compared to classical BSE in small ruminants. The efficient intraspecies transmissibility of ovine BSE via blood transfusion has been convincingly demonstrated by a number of consecutive transfusion experiments [19–22]. These experiments were also used as a model for the analysis of the putative transmission risks of the BSE-related human TSE designated variant CJD (vCJD)

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Abbreviations: BSE, Bovine Spongiform Encephalopathy; C-BSE, classical BSE; CJD, Creutzfeldt-Jakob disease; CWD, Chronic Wasting Disease; H-BSE, atypical BSE with higher MW of unglycosylated PrPC; L-BSE, atypical BSE with lower MW of unglycosylated PrPC; PMCA, Protein Misfolding Cyclic Amplification; TSE, Transmissible Spongiform Encephalopathy.

One supplementary table and figure are available with the online version of this article.

through blood transfusion [22–26]. While for sporadic CJD, neither PrP^{Sc} nor TSE infectivity have ever been detected in the lymphoreticular system or in circulating lymphocytes, both are easily detectable already before the onset of clinical symptoms in the case of vCJD [27, 28]. This has major implications regarding the safety of blood transfusions and surgical procedures, since no reliable preclinical test is available for the screening of blood donations for the presence of vCJD infectivity [24, 29, 30]. This issue has gained increased scientific and public health attention after the first cases of accidential vCJD transmission have been reported in humans after blood transfusion [30–33].

In contrast to this, only very little is known about the BSE transmission risk either by direct contact to blood from BSE-infected cattle or by intraspecies blood transfusion. Although transmission studies in conventional RIII mice did not indicate any BSE risk in the blood of preclinically or clinically BSE-affected cattle [34], the relatively low susceptibility of this mouse model to a BSE infection and the low volume of blood that can be tested in this assay severely compromise the interpretation of these results. Besides the impact this question has on the safety of animal handlers, especially veterinarians and abattoir staff, the question whether bovine blood products may contain BSE infectivity also has important implications for the risk assessment regarding medicinal and other products. Immediately after the detection of the first transfusion-related cases of vCJD, the possible risk of BSE transmission through bovine blood or blood components has been widely discussed [35]. This has resulted in the implementation of regulation 2011/C 73/01 by the European Commission regarding bovine blood products during the production process of medicinal products, as laid down in the 'Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01 rev.3)' in 2011 [36]. Since then, the production of medicinal products derived from bovine tissues is strictly regulated, and is restricted to tissues that are either retrieved from countries or regions with a negligible BSE risk (category A countries according to the WHO regulations), or tissues excluding category IA, which are defined in this regulation as 'high-infectivity tissues (central nervous system (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS)'. According to this note, category IA tissues and substances derived from them shall not be used in the manufacture of medicinal products, unless justified. This regulation also takes into account the age of the animal and the manufacturing process. Special emphasis is given in this note to bovine blood and blood products, which is also relevant for bovine serum products used widely in *in vitro* cell-culture work. The risk assessment regarding the safe production of these products should also consider the traceability, the geographical origin and the age of the donor animals, the used stunning method, as well as the possible reduction of TSE agents during manufacture.

Taking these considerations together, we decided to perform a blood-transfusion study in cattle. We used six donor cattle that were in the clinical end stage of classical BSE after experimental oral exposure, three donor cattle each of the atypical BSE forms H- and L-BSE that were in the clinical end stage of disease after intracerebral inoculation, as well as two mock-infected BSE negative donors, and transferred their blood into two recipient calves each. The animals were observed for 10 years before being euthanized and necropsied. Their brains were analysed using a BSE rapid test ELISA as well as the highly sensitive detection method of Protein Misfolding Cyclic Amplification (PMCA), all with negative results.

METHODS

Donor cattle

The donor cattle used in this experiment were retrieved from three groups of experimental BSE transmissions. Group A were seven Simmental cross cattle that had been orally challenged with 100 g of high-titre BSE-positive bovine brainstem material [2]. Upon the onset of definite clinical BSE symptoms and 24–48 h prior to euthanasia and necropsy, up to two litres (L) of whole blood were collected from each donor into a maximum of four blood-transfusion bags routinely used by public health services. The quantity of the collected donor blood depended on the feasibility to withdraw a large volume from a clinically BSE-affected animal under acceptable conditions regarding work safety and animal welfare. Depending on the availability, one or two bags of donor blood were transfused to two recipients each (12 recipients altogether), either on the same day, or kept at 4 °C overnight before carefully warming to >30 °C and transfusing to the recipient. In some instances, the blood of one donor could only be transfused into one animal (IT 64, IT 18), or only 0.5 l of donor blood could be transfused into one (recipient TR 04 from donor IT 59 and recipient TR 18 from donor RA 10) or both of the recipients (TR 19, TR 20 from donor RA 06).

Group B donors were retrieved from an intracranial transmission study of atypical H-BSE (three donors, six recipients), while group C donors belonged to an intracranial L-BSE transmission study (three donors, six recipients) [37]. Collection and storage of these blood-donation samples was performed as described for group A.

The blood of two negative-control donors was used for transfusion into two additional recipient animals. Altogether, the whole study comprised 26 recipient animals that received transfusions from cattle affected with C-, H- or L-BSE, or from negative-control cattle. The donors, their clinical status, the results of the immunohistochemical BSE diagnostic test, as well as the respective transfusion volumes and recipient animals are listed in Table 1.

Recipient cattle and blood transfusion

Twenty-six female Holstein-Friesian cattle at the age of 4–6 months were retrieved from local farms with no history of a recent BSE case. The blood transfusions were performed in a timely line with the development of clinical symptoms in the donor animals, and were performed between July 2007

Table 1. Blood donors and recipients. Whenever possible, 1 l of donor blood was transfused into two recipients each. In some instances, the blood of one donor could only be transfused into one animal (IT 64, IT 18), or only 0.5 l of donor blood could be transfused into one (recipient TR 04 from donor IT 59 and recipient TR 18 from donor RA 10) or both of the recipients (TR 19, TR 20 from donor RA 06). This table also shows the clinical score and IHC results of the obex samples from the donor cattle

Donor ID	BSE type	Clinical score donor (0–3)	obex IHC result donor (+/++/+++)	Donor blood result PMCA	Recipient ID	Transfusion vol.
IT 59	C	3	+++	neg	TR 01	1 L
IT 59	C	3	+++	neg	TR 04	0.5 L
IT 64	C	3	+++	neg	TR 02	0.5 L
IT 18	C	3	+++		TR 03	1 L
IT 63	C	2–3	+++	neg	TR 05	1 L
IT 63	C	2–3	+++	neg	TR 06	1 L
IT 40	C	2–3	+++	neg	TR 07	1 L
IT 40	C	2–3	+++	neg	TR 09	1 L
IT 27	C	2–3	++	neg	TR 08	1 L
IT 27	C	2–3	++	neg	TR 12	1 L
IT 54	C	3	+++	neg	TR 10	1 L
IT 54	C	3	+++	neg	TR 11	1 L
KT 73	neg. contr.	0	0	neg	TR 13	1 L
KT 67	neg. contr.	0	0	neg	TR 14	1 L
RA 05	L	0–1	+(+)	neg	TR 15	1 L
RA 05	L	0–1	+(+)	neg	TR 16	1 L
RA 10	H	1	+(+)	neg	TR 17	1 L
RA 10	H	1	+(+)	neg	TR 18	0.5 L
RA 06	L	1	++	neg	TR 19	0.5 L
RA 06	L	1	++	neg	TR 20	0.5 L
RA 14	H	2	++	neg	TR 21	1 L
RA 02	L	2–3	++	neg	TR 22	1 L
RA 02	L	2–3	++	neg	TR 23	1 L
RA 14	H	2	++	neg	TR 24	1 L
RA 15	H	2–3	++	neg	TR 25	1 L
RA 15	H	2–3	++	neg	TR 26	1 L

and January 2008. Then, 24–48 h prior to the transfusion procedure, negative-control blood samples were collected from all recipient animals. As a biological transfusion probe, 10 ml of donor blood and 10 ml of recipient blood were slowly mixed in a petri dish and observed for any signs of coagulation for 10 min. The transfusion was performed following the recommendations from Hunt and Moore [38] and Soldan [39], starting very slowly for the first 100 ml (approx. 1 l h^{-1}), which was followed by a close observation of the animal for 10 min without transfusion. If no abnormal reaction was observed, the remaining blood volume was transfused with a slightly increased flow of 2–3 l h^{-1} as long as the recipient animals did not show any signs of adverse reaction to the transfused blood. In general, all animals were to receive 1 l of whole blood from one donor animal. However, only 500 ml were available for transfusion into five of the recipients.

The animals were observed closely for at least 30 min after the transfusion for any signs of circulatory disturbances that might be due to adverse reactions to the transfused blood.

Clinical observation

Upon transfusion, the animals were kept free-ranging in a cattle facility for 10 years with feeding of hay, hay cobs, concentrates and minerals twice daily and water *ad libitum*. They were checked daily for any clinical abnormalities by technical staff, and a thorough clinical examination for the onset of BSE-related clinical symptoms was performed every 6 weeks from 24 months post-transfusion (months p.t.) until the end of the experiment by a veterinarian. This examination was developed according to the procedure suggested by Braun *et al*. [40] and Braun [41], and had already been applied during earlier BSE transmission experiments in cattle preformed in the same facility. In general, this examination included the reaction of the animals to tactile, optic and acoustic stimuli, as well as of the general behaviour and the coordination in motion. The first examination steps were done individually from the front of the animals that were fixed in the feeding lots, by first rapidly approaching the animals. Then the blink reflex was tested by moving one hand alternately in front of both eyes. The next test addressed the reaction to a soft tactile stimulus induced by a pen touching the region innervated by *N. facialis*, i.e. the cheeks, the forehead and the area around the corners of the muzzle. This was followed by a movement of the investigator's boot exactly in front of the animal's head, which is outside the center of the field of vision of cattle. This procedure was followed by the exposure of the animals to several flashes of light at irregular intervals. The examination was continued from behind the animals by carefully testing the individual reactions to a tactile stimulus of the hind legs with a broom ('broom test'), as well as their reaction to a sudden noise from behind (clapping of hands against the synthetic protective coverall worn by all staff). The reactions of the animals to the stimuli described above were rated from 1 (no reaction) to 4 (very strong reaction) in 0.5 steps. Finally, the overall body condition was scored between 1 (fully nourished, partly obese) to 4 (cachectic). The second part of the examination procedure addressed the general behaviour and the coordination of the motion. For that, each animal was individually released from the feeding lot and was animated to walk up the area behind the other animals, then turn around (to monitor the coordination) and then walk straight along the other animals and out into a covered paddock, with a bar put on the floor as an obstacle they had to cross on the way to the paddock. During this part of the examination, the general behaviour was rated from 1 (calm and relaxed) to 4 (nervous), the gait was rated from 1 (normal) to 4 (severely ataxic), and the passing of the obstacle was rated from 1 (calmly taking a normal step over the bar) to 4 (jumping, difficulty in keeping balance). The scores of the first four rounds of behavioural analyses were not included in the study to give the animals sufficient time to get accustomed to the procedure. Adding all scores together, the maximum score would have been 44. We defined any total score above 17 that was observed after more than 30 months p.t. (starting of the analyses at 24 months p.t. plus 6 months adjustment phase to the procedure) as preliminary suspicious of early BSE signs. The process of the behavioural analysis of the last examination at 120 months p.i. is depicted in Table S1 (available in the online version of the article).

Regular sampling throughout the experiment

Throughout the experiment, whole blood, plasma, serum and buffy coat were collected from each animal every 6 months from the time point of blood transfusion. Moreover, cerebrospinal fluid (CSF) was collected from the lumbosacral spatium every 12 months under sedation with 30 mg/100 kg xylazine. The analysis of these samples will be described elsewhere.

Necropsy and TSE testing of collected samples

The animals were sampled for whole blood, plasma, serum and buffy coat before premedication with 200 mg/100 kg xylazine and 200 mg/100 kg ketamine and euthanasia with 1200 mg/100 kg Embutramide (T61, Intervet International GmbH, Unterschleißheim, Germany). Then, at least 15 ml of CSF was collected from the lumbosacral spatium. During the necropsy, all organs were sampled under TSE sterile conditions. Tissue specimens were stored at −20/−80 °C for biochemical analyses and in formaldehyde for histological analyses.

First, the brainstem samples of all cattle were tested using a commercial BSE rapid test (TeSeE Test, BioRad Munich, Germany) according to the manufacturer's instructions. These brainstem samples were also analysed for seeding activity by PMCA as described below for the blood samples.

PMCA analysis of donor brain and blood samples and of transfusion recipient brain samples

All donor brain and blood samples as well as the recipient brainstem samples were tested for seeding activity by PMCA. Brain homogenates of bovine PrP transgenic mice (TgbovXV mice) served as a substrate, and 10% brain homogenates of the positive and negative controls as well as the recipient cattle were mixed in substrate in a 1:10 ratio into the 250 µl reaction

volume as described previously [42, 43]. One PMCA round of 24 h comprised 48 cycles of 20 sec sonication followed by 29 min 40 sec incubation at 35 °C. As a positive control, a C-BSE positive cattle brain homogenate in dilutions of 10^{-3}, 10^{-6} and 10^{-9} was used in three consecutive rounds of PMCA. We then added 50 µg ml^{-1} Proteinase K to each reaction and incubated at 55 °C for 1 h, which was followed by PTA precipitation and Western blot analysis as described previously [3]. The PMCA results were considered valid for C-BSE when a 10^{-3} and 10^{-6} dilution of the positive control animal were clearly positive. A 10^{-9} dilution was also always run, and the results for this dilution were expected to be at least borderline positive.

For H- and L-BSE, we used a slightly modified amplification protocol where one PMCA round covered 48 h comprising 48 cycles of 30 sec sonication followed by 59 min 30 sec incubation at 35 °C. H- and L-BSE-positive cattle brain homogenates from an earlier challenge study [37] in dilutions of 10^{-3}, 10^{-6} and 10^{-9} were used as positive controls, and the PMCA was considered valid if the 10^{-3} dilution was clearly positive and the 10^{-6} dilution was at least borderline positive.

In the next step, 10^{-3} dilutions of the donor brain samples were analysed using the described protocols. Given that a negative result or only weak signal was anticipated for the recipient brain samples, we analysed these in a 10^{-1} dilution, as opposed to 10^{-3} dilution for the donor animals.

In order to roughly quantify the inhibitory effect of blood in the PMCA reaction experienced by others [44] we mixed negative cattle blood 1:1 with 10^{-3} and 10^{-6} dilutions of a C-BSE-positive brain homogenate and used these as the starting material in a PMCA reaction. The resulting signal intensities were quantified using the VersaDoc Imaging system (BioRad, München) with the Quantity One software. We also analysed the effect of different blood components on the efficiency of the PMCA reaction by adding bovine BSE-negative EDTA blood, plasma and buffy-coat samples spiked in a 1:1 ratio with a 10^{-3} dilution of a C-BSE-positive cattle brain and mixed these 1:10 with substrate in a PMCA reaction as described above.

In order to maximize the blood volume for the analysis of the donor-blood samples, we applied the purification protocol published by Concha-Marambio *et al.* [45]. To prove the sensitivity of this protocol, we spiked 500 µl blood volume with 2.5 µl of a 10^{-3}, 10^{-6} or 10^{-9} dilution of a C-, H- or L-BSE-positive cattle, mixed it with 500 µl of a 20% sarkosyl solution and incubated for 10 min at room temperature. Samples were then centrifuged at 100000 ***g*** for 1 h at 4 °C before the supernatant was discarded and the pellet was washed in 500 µl of PBS. Samples were centrifuged again at 100000 ***g*** for 30 min at 4 °C and the pellets were resuspended directly in 250 µl Tgbov XV substrate and used as the starting material for three consecutive PMCA rounds as described above for classical and atypical BSE. Finally, 500 µl of the donor blood samples were analysed using the above described purification and PMCA protocols.

RESULTS

PMCA protocol adaptation for the detection of atypical BSE seeing activity and PMCA analysis of donor brain samples

The PMCA protocol for C-BSE (48 cycles each 20 sec every 30 min) and the optimized protocols for atypical BSE (48 cycles each 30 sec every 60 min) allowed us to detect seeding activity in the positive control samples for all three BSE forms in dilutions down to 10^{-9}, while the negative control samples gave negative results (Fig. 1a). However, the PMCA for atypical BSE is not clearly quantitative, as we repeatedly detected signals in the first round of amplification that were decreased in the second round and increased again in the third round of amplification.

When we applied these amplification protocols, we were able to detect seeding activity in the brain samples of all C-, H- and L-BSE donor cattle (Fig. 1b), for C-BSE the donors IT 59, IT 40 and IT 64 are exemplarily shown.

Analysis of the inhibitory effect of blood components on the PMCA reaction efficiency and PMCA analysis of donor brain and blood samples

We then quantified the effect of the presence of blood mixed 1:1 with a 10^{-3} dilution of the C-BSE positive brain tissue control sample, and added these in a 1:10 ratio in the PMCA reaction. We found a 10% reduction when using a 10^{-3} dilution of the positive control and a 38% reduction when using a 10^{-6} dilution of the positive control (Fig. 2). To analyse the potentially inhibitory effect of different blood components on the efficiency of the PMCA reaction, we also performed experiments mixing again equal volumes of BSE negative EDTA blood, blood plasma or buffy coat into the reaction as described above. This setup did not result in a reduced seeding activity as compared to the positive control mixed 1:1 with Tgbov XV substrate (Fig. S1a). Next, we purified samples of 500 µl BSE negative EDTA blood, blood plasma or buffy coat that was spiked with the C-BSE positive control in 10^{-3}, 10^{-6} and 10^{-9} dilutions using a published centrifugation protocol [45]. All PMCA reactions revealed positive signals with comparable seeding activities that were slightly reduced as compared to the positive control 10^{-3} without addition of blood components (Fig. S1b).

We then proceeded to perform PMCA of negative blood samples spiked with a C-BSE positive control brain in 10^{-3}, 10^{-6} and 10^{-9} dilutions, and showed that the presence of blood in the PMCA reaction did result in a slight reduction of sensitivity, but we were still able to detect the 10^{-6} dilution of the positive control sample spiked into the blood sample (Fig. 3a). Next, we performed the same experiment using dilutions of H- and L-BSE spiked into negative blood and applied the PMCA protocol for atypical BSE. We were able to prove that the presence of blood in the atypical BSE PMCA reaction did not have a strong inhibitory effect on the reaction efficiency of H-BSE (Fig. 3b) or L-BSE (Fig. 3c).

In the next step, we continued with the analysis of the donor blood samples. The blood samples of the C-donors were

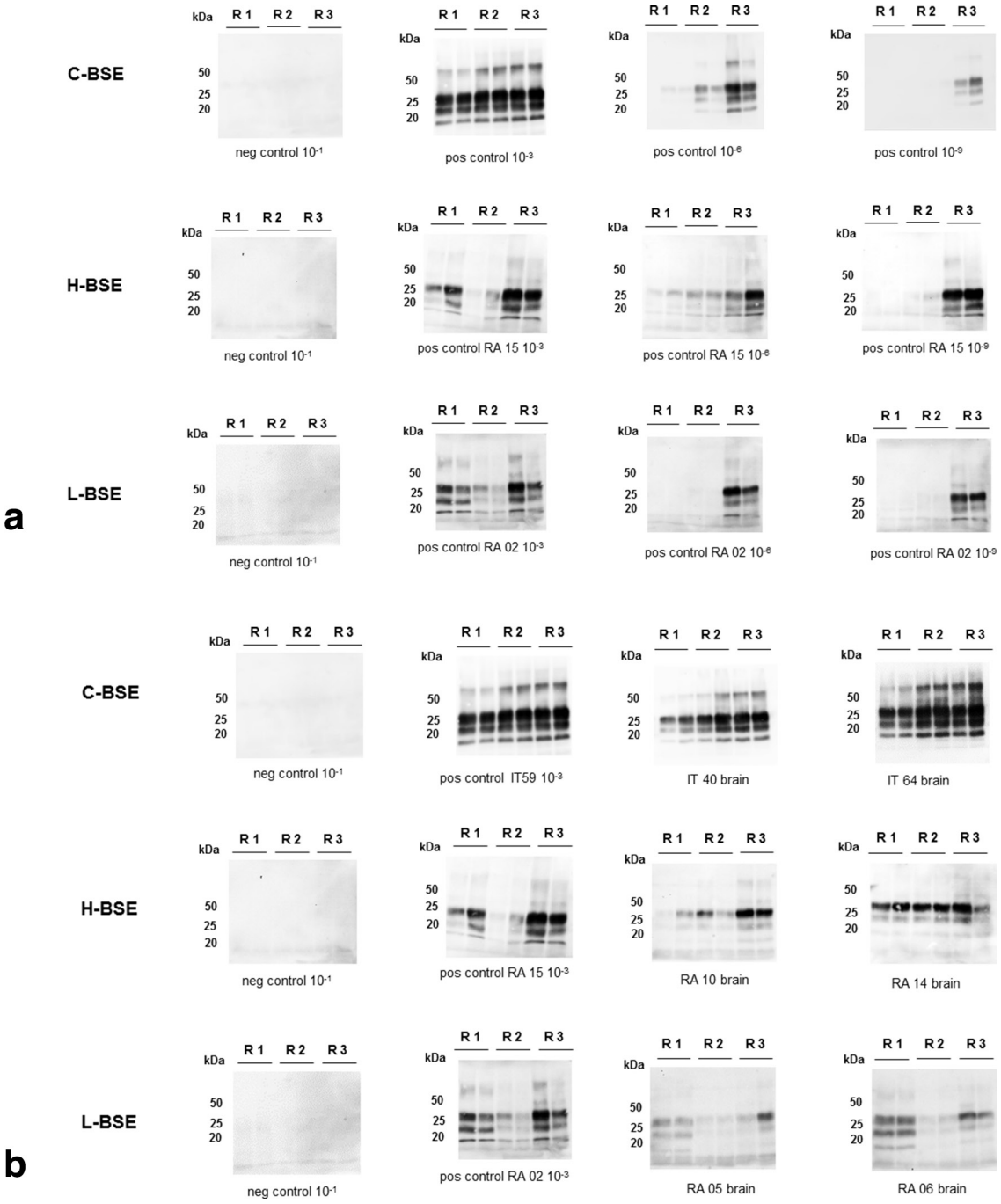

Fig. 1. PMCA protocol adaptation for the detection of atypical BSE seeding activity and PMCA analysis of donor-brain samples. (a) C-, H- and L-BSE positive brainstem samples used as positive controls gave three clear banding signals specific for amplified PrP^{res} down to a dilution of 10^{-9}. Three rounds of amplification were performed in duplicate, marked as R1, R2, R3. (b) Donor brain samples were all positive (for the C-BSE donors, results for IT 59, IT 40 and IT 64 are exemplarily shown).

negative, with the exception of donors IT 18 and 54, where a weak seeding activity was detectable in one of the duplicates (Fig. 3a). Analysis of the blood samples of all H-BSE (Fig. 3b) and L-BSE (Fig. 3c) donor cattle confirmed the positive result for the brain samples, while all donor blood samples were negative.

Blood transfusion

All 26 blood transfusions were performed without any complications. None of the animals developed any adverse reaction during or immediately after the transfusion process.

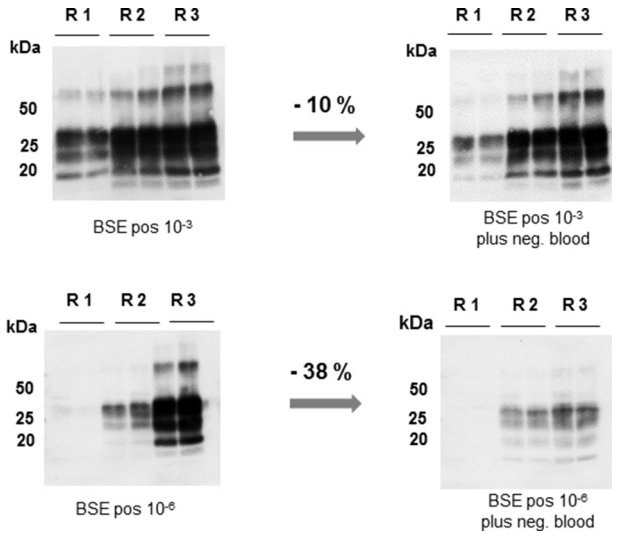

Fig. 2. Quantification of the inhibitory effect of the presence of blood in the PMCA reaction by mixing equal volumes of positive brain homogenate and blood into the PMCA reaction. A 10^{-3} or a 10^{-6} dilution of a C-BSE positive brain homogenate with and without the addition of an equal volume of BSE negative bovine blood was used as the starting material in a PMCA reaction. Three rounds of amplification were performed in duplicate, marked as R1, R2, R3. The resulting signal intensities were quantified using the VersaDoc Imaging system (BioRad, München) with the Quantity One software.

Clinical observation

After the initial phase of the animals adjusting to the examination procedure, the behavioural scores of all animals were below the defined threshold of 17 points. This threshold was defined by assuming that a BSE suspicious animal would give clearly elevated scores of 3 or higher in at least 3 out of the 11 categories. In some instances, individual elevated scores were noted for the gait due to BSE-unrelated lameness (e.g. sole ulcer). Even including these BSE-unrelated increases in the clinical score, no total scores higher than 15 points were observed throughout the study. The individual scores of the last examination per animal are displayed in Table S1, the summary of all clinical observations is shown in Fig. 4.

Some of the animals had to be euthanized for animal welfare reasons due to unrelated disease in the course of the 10 year experiment. These were TR 07 (101 months p.t.) and TR 08 (98 months p.t.) due to sole ulcers, TR 19 (86 months p.t.) due to an indigestible foreign body, TR 22 (83 months p.t.) due to a progressive and severe loss of body condition without a know reason (also after necropsy), and TR 26 (95 months p.t.) due to a chronic mastitis that was unresponsive to antibiotic and anti-inflammatory treatment.

Rapid test and PMCA analysis of brainstem samples of recipient animals

The brainstem samples of all recipient animals gave clearly negative results in the applied BSE rapid test (TeSeE Test, BioRad, Munich, Germany). We then tested these samples for their seeding activity by PMCA, again all with negative results, while the C-BSE-positive control showed a clear seeding activity down to a dilution of 10^{-9} and the atypical BSE positive control samples showed weaker but detectable seeding activity at dilutions of 10^{-3} and 10^{-6}. The results of the PMCA analyses of the brainstem samples are exemplarily shown for one recipient of each BSE form in Fig. 5.

DISCUSSION

This blood transfusion experiment from cattle clinically affected with either classical (C-BSE) or atypical BSE

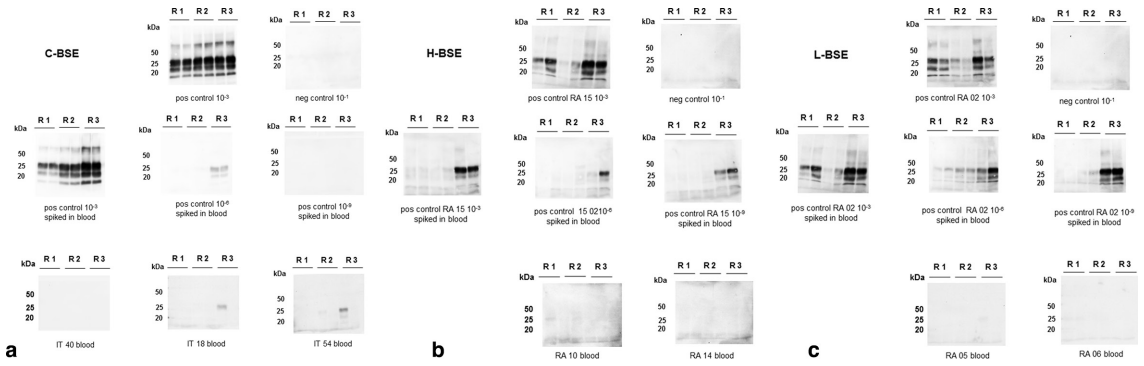

Fig. 3. PMCA analysis of donor blood samples. (a) The blood purification protocol of a negative blood sample spiked with different dilutions of the C-BSE-positive brainstem sample used as a positive control resulted in positive signals in the 10^{-3} and the 10^{-6} dilution. The donor blood samples of IT 27, IT 40, IT 63, IT 64 and IT 59 were negative as exemplarily shown for IT 40, while the blood samples of IT 18 and IT 54 repeatedly gave a weak positive result in one duplicate. (b) Detectable seeding activity after blood purification spiked with dilutions of H-BSE even in the 10^{-9} dilution. The donor blood samples of the three H-BSE donors in a 10^{-1} dilution gave negative results. (c) Detectable seeding activity after blood purification spiked with dilutions of L-BSE even in the 10^{-9} dilution. The donor blood samples of the three L-BSE donors in a 10^{-1} dilution gave negative results. Three rounds of amplification were performed in duplicate, marked as R1, R2, R3.

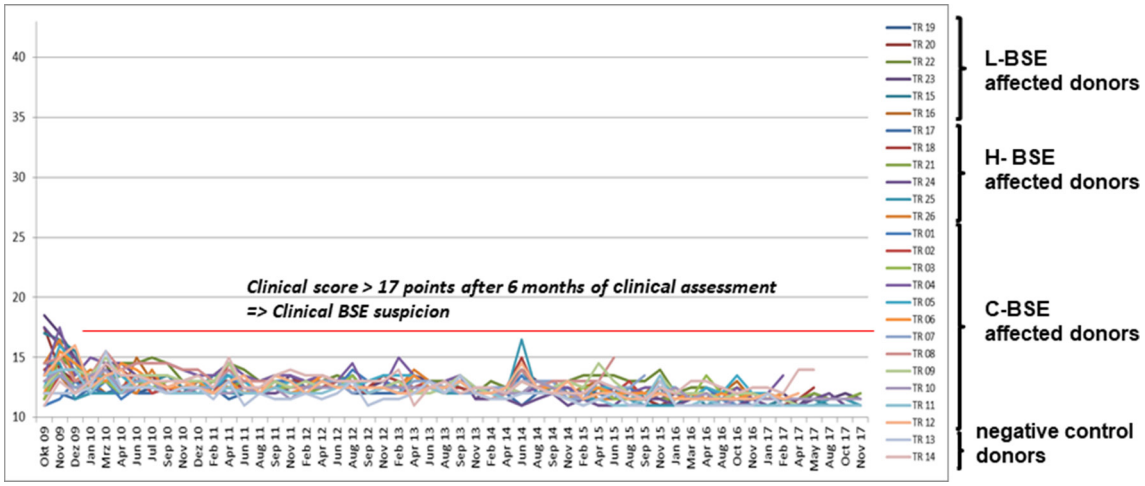

Fig. 4. Behavioural analysis of BSE blood recipients between 36 and 120 months p.t. Clinical scores of 0–4 in the 11 categories that were assessed during the clinical observation procedure added to a theoretical total of 44 points. In total, 17 points were defined as a threshold for a clinical BSE suspicion, as this number would have been reached, in case an animal reached a score of 3 in at least three categories.

(H- or L-BSE) is the first intraspecies transmission experiment addressing the assessment of BSE infectivity in the circulating blood of bovines in the clinical end stage of the disease. The donor animals, which were showing clear clinical signs of BSE, had accumulated a profound load of PrP^{Sc} and BSE infectivity in their CNS, and were therefore considered ideal donors for an experiment assessing the possible presence of BSE infectivity in their circulating blood. The PMCA protocol had recently been optimized for the analysis of even borderline C-BSE-positive bovine samples [42, 43], and was shown to harbour a comparable diagnostic sensitivity to the bovine PrP transgenic Tgbov XV mouse bioassay, which made

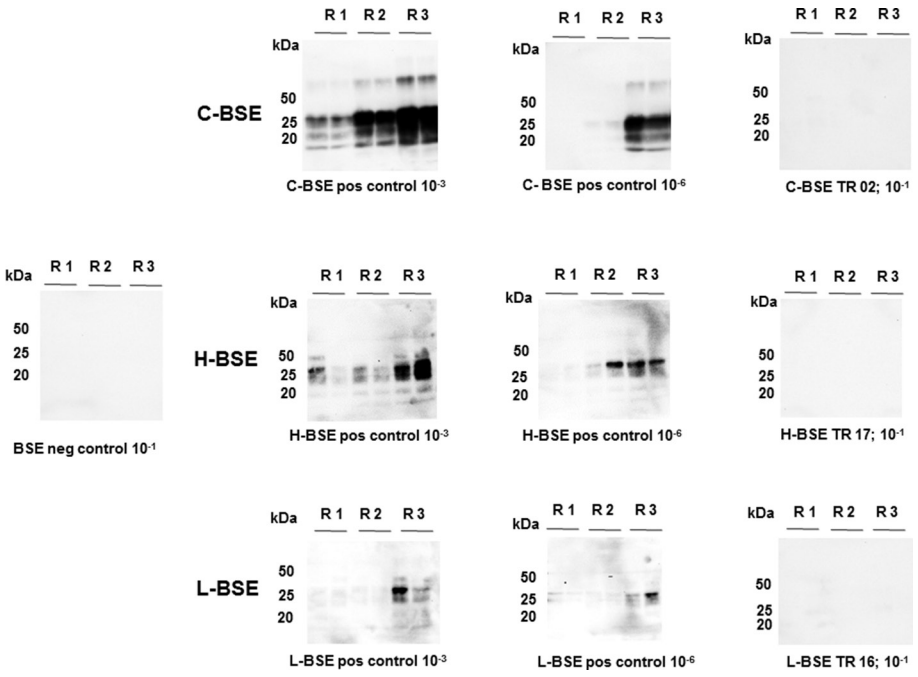

Fig. 5. PMCA analysis of brainstem samples from recipient animals. Three rounds of amplification were performed in duplicate, marked as R1, R2 and R3. While the C-, H- and L-BSE-positive brainstem samples that were used as positive controls gave positive results at dilutions of 10^{-3} and 10^{-6}, all recipient cattle were negative. This figure exemplarily shows one recipient animal per BSE form (C-, H- and L-BSE).

the additional analysis of these samples by transgenic mouse bioassay dispensable. In this project, we optimized the PMCA protocol for the detection of seeding activity in atypical BSE samples. The complexity of the detection of atypical BSE by PMCA has already been stated in earlier reports of the amplification of L-BSE [46] and H-BSE [47]. After adaptation of our PMCA protocol for the amplification of atypical BSE, we were still able to show a signal amplification using 10^{-3}, 10^{-6} and 10^{-9} dilutions of atypical BSE- (H- and L-BSE) positive brain homogenates, although with a slightly lower repeatability as compared to C-BSE. This protocol allowed the analysis of the brain and blood samples of the atypical BSE donor and transfusion recipient animals. Although this assay seems to be less quantifiable than the assay used for the amplification of C-BSE, we were able to show that it is still suitable for the reliable detection of seeding activity in atypical BSE samples.

To also take into account the inhibitory effect of blood on the PMCA reaction, we first added blood components to the PMCA reaction 10^{-3} and 10^{-6} dilutions of the positive control with bovine blood, which did result in a signal reduction of 10% and 38%, respectively, but the sample was still clearly detectable as positive. We then proceeded to using 500 µl of a negative blood sample and showed that even in this setup the positive control sample was still clearly detectable at a 10^{-6} dilution. When applying this protocol, the analysis of all donor blood samples (C-, H- and L-BSE) by PMCA did not reveal a BSE seeding activity, with the exception of two C-BSE donors (IT 18 and IT54) where we detected a weak seeding activity signal in one duplicate in the third round of amplification. However, even these blood samples did not contain sufficient amounts of BSE infectivity to induce a progressive BSE infection in the recipient animals even after 10 years.

Although the blood-group system in cattle is highly complex and exceeds the complexity of the human blood-group system [48–50], adverse effects after blood transfusion are generally rare [38, 39, 51]. This is due to the fact that bovine red blood cells do not agglutinate easily, and the important factors in transfusion reactions are haemolysins. Pre-formed isohaemolysins are normally lacking or present only at very low quantities in bovine serum [38]. Consequently, initial unmatched blood transfusions can be given without a serious threat of a fatal reaction. This was confirmed in our experiment where the transfusion of up to 1 L of whole blood from donor to recipient cattle did not cause any adverse reactions in the recipient animals.

The clinical assessment procedure described in this paper has already been applied for the examination of the donor animal groups [2, 37], proving its reliability by detecting early clinical sings of a BSE infection in these BSE positive animals. In this group of recipient animals, no animal gave any clinical indications of a BSE infection. However, individual scores were elevated, especially the gait score when animals had episodes of lameness due to unrelated reasons (e.g. sole ulcer). Also, a general agitation within the herd related to the oestrus cycles of the animals was noticeable, but never resulted in total scores exceeding the threshold of 17 points. The results of this clinical assessment procedure from two positive [2, 37] and one negative (this study) groups of cattle confirms the excellent reliability of these criteria for the clinical assessment of possibly BSE related symptoms. This procedure is also easily applicable in the field, where it was originally developed as a recommendation for practitioners working on cattle farms or slaughterhouses [40, 41].

The post-mortem evaluation of the recipient animals was first performed by using an approved BSE rapid test. However, since the negative results of the rapid test might be interpreted as questionable due to the relatively low sensitivity of this assay, we also analysed the brainstem samples for their seeding activity by PMCA. All PMCA analyses also yielded negative results, adding further data regarding the extremely low level (which due to technical restraints of the diagnostic assays cannot be completely ruled out in the case of atypical BSE) or even absence of BSE infectivity in the blood of cattle in the clinical end stage of classical or atypical BSE.

Taken together, these results add important information enabling a reassessment of the BSE contamination risk of bovine blood and blood products used for the production of medicinal products and other preparations. According to current regulations, bovine blood products should always be retrieved from countries with a negligible BSE risk, and the traceability, geographical origin, the age of the donor animals, the used stunning method, as well as the possible reduction of TSE agents during manufacture need to be taken into consideration during the risk assessment of the intended product. This makes the production, approval and commercialization of bovine blood products, medicinal products or other preparations containing bovine blood products (including fetal or newborn calf serum widely used in cell culture work) extremely costly and inefficient. In light of the results presented here, lightening these strict regulations should be considered for the preparation of bovine blood and blood products, as the level of BSE infectivity in blood products of cattle in the clinical end stage of disease is either below the detection limit of our tests (atypical BSE), or, according to present knowledge, is completely absent (C-BSE).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All infection experiments in cattle described in this paper were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany (LALLF 7221.3-1-025/13/3), on the basis of national and European legislation, namely the EU directive 2010/63/EU for the protection of animals used for experiments.

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