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A prion reduction filter does not completely remove endogenous prion infectivity from sheep blood

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Abstract

Background

Variant Creutzfeldt-Jakob disease (vCJD) is a transmissible spongiform encephalopathy (TSE) affecting humans, acquired initially through infection with bovine spongiform encephalopathy (BSE). A small number of vCJD cases have been acquired through the transfusion of blood from asymptomatic donors who subsequently developed vCJD. Filter devices that selectively bind the infectious agent associated with prion disease have been developed for removal of infection from blood. This study independently assessed one such filter, the P-CAPT filter, for efficacy in removing infectivity associated with the BSE agent in sheep blood. The sheep BSE model has previously been used to evaluate the distribution of infectivity in clinically-relevant blood components. This is the first study to assess the ability of the P-CAPT filter to remove endogenous infectivity associated with blood components prepared from a large animal model.

Methods

Paired units of leucoreduced red cell concentrates (LR-RCC) were prepared from donors at the clinical stage of infection and confirmed as having BSE. One cohort of recipients was transfused with LR-RCC alone, whereas a parallel cohort received leucoreduced and P-CAPT-filtered RCC.

Results

Out of fourteen recipients, two have been confirmed as having BSE. These sheep had received leucoreduced RCC and leucoreduced/P-CAPT-filtered RCC from the same donor.

Conclusions

The results indicate that, following leucoreduction and P-CAPT filtration, there can still be sufficient residual infectivity in sheep RCC to transmit infection when transfused into a susceptible recipient.

Keywords

Prions, prion filtration, leucoreduction, blood transfusion, red cell concentrates, P-CAPT

Introduction

The identification of variant Creutzfeldt-Jakob disease (vCJD) in humans in 1996[1,2] is believed to be linked to the consumption of meat products from cattle infected with bovine spongiform encephalopathy (BSE).[3] The UK has shown the highest incidence of vCJD in the world with 177 definite or probable cases reported up until 3rd November 2014.[4,5] During the early stages of the vCJD outbreak, the risk of disease transmission *via* iatrogenic routes such as blood transfusion, organ donation and surgical procedures was unknown. However, data from multiple prion-infected models were beginning to show that blood collected before the onset of clinical signs could transmit infection following experimental inoculation of laboratory hosts.[6-13] This was confirmed using large animal models (sheep and deer), which have provided convincing evidence that blood transfusion is also an efficient route of prion transmission.[14-20]

The Transfusion Medicine Epidemiology Review (TMER) was set up in 2006 to investigate whether there was evidence that Creutzfeldt-Jakob disease (CJD) or vCJD may have been transmitted *via* the blood supply.[21] This review has identified three cases of vCJD[21-26] in UK residents who had been transfused with non-leucodepleted red cell concentrates (RCC) from donors who were later confirmed to have died from vCJD. In common with all confirmed clinical cases of vCJD to date, these individuals were homozygous for methionine (M) at codon 129 of the *PRNP* gene (129MM). A fourth recipient of non-leucodepleted RCC, who died of causes unrelated to vCJD, but tested positive for disease-associated PrP (PrP^d) and infectivity in the spleen, was methionine/valine (M/V) heterozygous at codon 129 (129MV).[27] More recently, a 129MV haemophiliac patient who had received Factor VIII prepared from plasma pools known to contain donations from a vCJD donor, also tested positive for PrP^d in spleen samples, but again had no history of neurological disease.[28] The latter two cases raise the possibility that individuals with 129MV and 129VV genotypes are also susceptible to infection with vCJD, but may have longer incubation periods or be asymptomatic carriers of infection. This is supported by data from infection of mice transgenic for the human *PRNP*

gene.[29] Moreover, recent large-scale surveys of human appendices identified samples from individuals of 129MM, 129MV and 129VV genotypes positive for PrP^d by immunohistochemistry, resulting in prevalence estimates for sub-clinical vCJD infection of up to 1 in 2000 of the UK population.[30] In the absence of a validated diagnostic test to screen for vCJD infection, these asymptomatic individuals present a potential risk for further human-to-human transmission *via* iatrogenic routes.

To reduce the likelihood of vCJD transmission *via* blood products, a number of protective measures have been adopted in the UK, including donor deferral, importation of plasma, and leucoreduction (filtration to remove white blood cells) of all components used in human transfusion medicine.[31,32] Continued follow-up in the TMER study has not yet identified any evidence of infection in patients who received leucoreduced blood components from known vCJD cases. However several experimental models have shown that not all endogenous infectivity in blood is removed following leucoreduction.[17,33,34] Therefore additional risk reduction measures have been considered for the targeted removal of the infectious agent (PrP^d) from human blood components prior to transfusion or preparation of plasma-derived therapeutic products.[35-38] One novel approach is prion filtration, which aims to remove abnormal PrP from blood by selective binding to affinity ligands or resins, using devices such as the MacoPharma P-CAPT filter,[33,39,40] the Pall Corporation Leukotrap filter[41-43] and the Asahi KASEI combination filter.[42] Many of the studies evaluating the efficacy of these prion reduction filters have used brain spiked into blood or blood components[39-41,43] as a way to assess the extent of prion removal. However, this approach may not replicate the effects of filtration on endogenous infectivity in blood.

The aim of this study was to assess the efficacy of a specific prion removal filter, the CE marked P-CAPT filter, in removing endogenous infectivity associated with the BSE agent from sheep blood. The effects of the filter have previously been tested using both spiked[40] and endogenous infectivity[33] associated with the 263K hamster scrapie strain. Studies to

assess the effect of processing human leucoreduced red cell concentrates, using the P-CAPT filter, identified no issues surrounding the clinical safety of P-CAPT-filtered products[44] and found no evidence of adverse effects with respect to component storage, stability, viability or other measures of red cell quality.[45-48]

We have applied the P-CAPT filter to leucoreduced units of red cell concentrates prepared from sheep infected with BSE and compared the qualitative effects, in terms of disease transmission and detection of abnormal prion protein, with the transfusion of paired units of leucoreduced red cells alone. In this sheep model, the distribution of both infectivity and of abnormal prion protein in peripheral lymphoid tissues is similar to that of humans affected with vCJD.[49,50] The relevance of this model in assessing the transfusion-related transmission of prion diseases in sheep, has been documented in several publications.[14-17] We have previously shown the presence of infectivity in both red cell concentrates and leucoreduced equivalents, when blood was collected from sheep at a pre-clinical stage of infection. In this study, we report on infectivity associated with leucoreduced red cell concentrates that have been filtered using the P-CAPT filter.

Materials and methods

Experimental animals

The animal experiments were approved by The Institute for Animal Health and The Roslin Institute's (University of Edinburgh) Animal Welfare and Ethical Review Committees and experiments conducted according to the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986.

The sheep were sourced from the DEFRA scrapie-free flock[51] and were ARQ/ARQ at codons 136, 154 and 171 of the sheep *PRNP* gene (genotypes determined as previously described[15]). The genotypes at codon 141 are indicated in Table 1, whereby the effects relate to survival times associated with experimental scrapie and BSE infection [52,53].

Donor sheep and blood collection

For this study, seven sheep were used as blood donors. This cohort was part of a larger group of sheep used as blood donors in an ongoing blood transfusion study (referred to as the leucodepletion study), and were maintained as previously described.[17] Donors were orally challenged with 5 g of brain homogenate, prepared from BSE-infected cattle (our reference BBB4/5, AHVLA reference SE1909/BBP2) and were clinically monitored until they reached defined humane clinical endpoints associated with BSE infection, as previously described.[15] Immediately before euthanasia, two units of whole blood (WB, 1 unit = 450 ml ± 10% (v/v)) were collected from each donor into quadruple, bottom and top (BAT) blood packs (Fresenius Hemocare, NPBI) fitted with in-line leucoreduction filters, containing 63 ml of anti-coagulant CPD.A1 (citrate, phosphate, dextrose, adenine).

Preparation of leucoreduced red cell concentrates (LR-RCC) and leucoreduced and P-CAPT-filtered red cell concentrates (LR-RCC-P-CAPT)

The methods of preparing blood components, including P-CAPT-filtered equivalents for transfusion were written in conjunction with staff at the Scottish National Blood Transfusion Service (SNBTS) to mimic procedures used for the processing of blood from patients and to achieve compliance with instructions provided for use of the prion reduction filter. An illustrative figure showing the blood collection and component preparation process is shown in supplementary figure S1. Immediately after donation (day 0), the 2 whole blood units were processed to red cell concentrates, as previously described.[17] Red cells were leucoreduced (LR-RCC, using the inline T3953 filter) were stored overnight at 4 ºC (without shaking).

The following day (day 1), both LR-RCC units were warmed to 22 ºC and mixed gently using a see-saw shaker for 30-60 minutes to ensure homogeneity. The two LR-RCC units were then pooled into an oversized blood bag with tubing connected to two separate transfer bags (referred to as the Christmas tree configuration pack). This approach was employed to make

each of the final products to be transfused as homogenous as possible not only in terms of their cellular content but also in terms of the distribution of infectivity associated with the BSE agent in blood. After pooling and gentle mixing to ensure homogeneity, the pooled LR-RCC was allowed to flow into the transfer packs, resulting in two separate units of LR-RCC of approximately equal volume. One of the two LR-RCC units was transfused to a recipient sheep without further processing, following cross-matching of donor and recipient blood samples, as previously described.[15]

The other LR-RCC unit was processed using the P-CAPT filter according to the manufacturer's instructions. A P-CAPT filter and associated transfer pack was docked (using sterile conditions) to the LR-RCC unit, with tubing separating the two (of a standard length of 15 cm). A stainless steel adjustable clamp was fitted to the tubing immediately below the P-CAPT filter and closed completely prior to filtration. This was used to manage the flow rate of blood during the filtration process. The snap valve fitted inside the tubing between the LR-RCC bag and the P-CAPT filter was opened to allow the flow of LR-RCC into the P-CAPT filter. Next, the adjustable stainless steel clamp was opened by a few (4-5) millimeters allowing controlled flow of LR-RCC through the P-CAPT filter and into the associated transfer pack, with the aim of ensuring that the filtration time took between 40 – 60 m (in line with recommendations from the manufacturer relating to blood contact time with the P-CAPT filter). The resultant LR-RCC-P-CAPT component was transfused to a recipient sheep, following cross-matching of donor and recipient blood samples.

Specifications of LR-RCC and LR-RCC-P-CAPT components used for transfusion

The volumes of each unit of whole blood collected, LR-RCC and LR-RCC-P-CAPT components prepared (ml) were determined by subtracting the weight of an empty collection bag from the filled weight of each component and dividing by the specific gravities of 1.06 for whole blood and red cell concentrate, as previously described.[17] Similarly, the methods for determining leucocyte counts and the distribution of plasma in the RCC, LR-RCC and LR-

RCC-P-CAPT components have already been described in detail.[17] The time (in minutes) taken for leucoreduction and P-CAPT filtration was also recorded.

Leucocyte evaluation using quantitative real-time PCR of sheep CD45

CD45 antigen is a universal and specific marker of leucocytes in blood. In addition to the BD Leucocount technique previously described,[17] residual numbers of leucocytes from sheep blood samples were determined by quantitative real-time PCR (qPCR) of CD45 genomic DNA (gDNA) by two separate methods using an Applied Biosystems 7000 Sequence Detection System (Life Technologies). Small volume aliquots (0.5 – 1ml) of each unit of RCC (prior to leucoreduction), LR-RCC and LR-RCC-PCAPT were stored at -20 ˚C at the time of preparation. gDNA was extracted from 50 µl of sheep blood using the Invitrogen ChargeSwitch gDNA Sheep Blood Kit (Life Technologies), and two PCR methods were used to quantitate gDNA, Power SYBR Green PCR (Applied Biosystems, Life Technologies, Paisley, UK) and Taqman Universal PCR (Life Technologies), according to the manufacturer's protocols. The primers were designed using the mRNA sequence of sheep CD45, which was identified from a blast search of the sheep genome v2.0 http://www.livestockgenomics.csiro.au/sheep/oar2.0.php against the bovine homologue of human CD45 (NM_001206523.1). The forward primer was 5'-GCACCTACATCGGCATCGA, and the reverse primer 5'-CGTAGACGTCCACCTTGTTCTCT. The number of leucocytes in each sample was calculated from the amplified gDNA by comparison against a standard curve of qPCR amplified CD45 from samples containing gDNA of known concentration (0.6 pg - 60 ng) assuming one cell contained 6.6pg of DNA.

Recipient sheep

As for donor sheep, recipient sheep were maintained and monitored for clinical signs of BSE infection until they reached defined humane end points or were culled for other reasons. Samples of recto-anal mucosa-associated lymphoid tissues (RAMALT) were collected at intervals to monitor for pre-clinical infection, as previously described.[54] At post mortem,

samples of brain and lymphoid tissues (tonsil, spleen, ileal Peyer's patch, mesenteric lymph node, prescapular lymph node) were collected and either fixed in neutral buffered formalin or frozen at -80 ^{o}C.

Detection of disease-associated PrP in tissues by Western blotting and immunohistochemistry

Proteinase K (PK)-resistant PrP^{Sc} in brain and lymphoid tissues was detected using established Western blot methods, probing with the monoclonal antibody (mAb) ROS-BC6 (0.5 µg/ml) as previously described.[17,55,56] Hyperfilm (GE Healthcare, Buckinghamshire, UK) was scanned and the digital image processed using Adobe Photoshop. Image processing was restricted to cropping, preparation of composite figures and annotation.

Formalin-fixed sections of brain and lymphoid tissues were also analysed for PrP^{d} deposition by immunohistochemistry using mAbs ROS-IH9[56] and BG4, as previously described. The monoclonal antibodies were applied and incubated overnight - ROS-IH9 (0.5 µg/ml at 4^{o}C) or BG4 (1.25 µg/ml at ambient temperature). Each assay included known BSE positive and negative control tissues sections to verify the sensitivity and specificity of the procedure. Immunohistochemical staining of rectal biopsy samples were performed as previously described, using the mAb R145.[54,57]

Results

Confirmation of BSE infection in blood donors

The seven sheep selected as blood donors for this study showed clinical signs typically associated with BSE infection, including pruritus and ataxia, with incubation periods ranging from approximately 800 to 1100 days. Brain and peripheral lymphoid tissues from each donor tested positive for the presence of abnormal prion protein by Western blotting and immunohistochemistry. Figure 1 (A-C) shows positive staining in the dorsal motor nuclei of the vagus nerve (DMNV) of three donors with widespread, diffuse extra-cellular PrP^{d}

labelling evident in each animal. The staining pattern is consistent with that seen in the positive control section (known BSE sheep sample, panel H) and distinct from that seen in the negative control section (known uninfected control sample, panel I), where no PrP^d labelling is evident. Two antibodies were used to confirm and validate the experimental outcomes (figure S2). The same PrP^d staining profile was observed in the other BSE positive donors (not shown).

Transfusion components meet required specifications

Table 2 show the parameters measured for each unit of whole blood collected from the donors and when processed to LR-RCC and LR-RCC-P-CAPT, including volumes and filtration times. Other parameters, such as the haematocrit and percentage of plasma in different components, were measured to assess the consistency of components prepared from different donors (data not shown), and did not differ significantly from those previously reported.[17]

Following leucoreduction, the number of leucocytes in each paired unit of LR-RCC and LR-RCC-P-CAPT was measured by flow cytometry and by quantitative real time-PCR for the CD45 gene expressed by all leucocytes. Table 2 shows the residual number of leucocytes in the LR-RCC and LR-RCC-P-CAPT units respectively, determined using the cytometer method. All of the units tested contained less than 1×10^6 leucocytes / unit, and thus met the specification for human leucoreduced blood components.[58] The results of the CD45 qPCR were similar for samples from all donors, with estimated numbers of leucocytes approximately 3 logs less than that seen in the RCC units (data not shown), providing confirmatory evidence of adequate leucoreduction in units where it was not possible to obtain a leucocyte count by cytometry (donors N220, N259).

Infectivity associated with P-CAPT-filtered leucoreduced red cells

Table 3 shows the timing of and outcome of RAMALT biopsies in euthanized and surviving transfusion recipients. The only recipients found to have positive staining for abnormal PrP^{d} in lymphoid follicles within biopsies were P524 and P511, which received paired LR-RCC and LR-RCC-P-CAPT components (respectively) from donor N220. Sheep P511 had three positive biopsy results from samples collected between 676 and 942 days post infection (representing 64% - 90% of the interval between transfusion and post mortem = survival period), while P524 had a single positive biopsy, which was collected close to the time when it developed clinical signs (92% of survival period). No further biopsies were undertaken as it has been observed that with age and in the absence of antigenic stimulus the follicles regress and become more scarce (data not shown).

The same two sheep (P524, P511) were later euthanized following the demonstration of definite or suspect clinical signs associated with BSE. Two additional recipient sheep (P548, P467) were culled for welfare reasons. Immunohistochemistry and Western blotting performed on brain and lymphoid tissue samples confirmed that P524 and P511 were infected with BSE, while P548 and P467 were not. Figure 1 shows the pattern of abnormal prion protein deposition in the DMNV from recipient sheep P524 (panel G) and P511 (panel D), which is consistent with seen in their donor (N220, panel A), and similar to that previously reported for sheep experimentally infected with BSE. Figure 2 shows Western blots of PK-resistant PrP^{Sc} prepared from brain and lymphoid tissues from the clinically positive (P524, P511) and clinically negative (P467, P548) sheep. PK-resistant PrP^{Sc} was identified in brain and most lymphoid tissues tested in P524, and in brain, spleen and ileal Peyer's patch of P511. No PK-resistant PrP^{Sc} was detected in brain or lymphoid tissue in recipients P467 or P548 both of which received leucoreduced red cell concentrates from donors N245 and N218 respectively. Taken together, the results confirm that the only transfusion recipients that have shown clinical and/or pathological evidence of infection are

P524 (LR-RCC recipient) and P511 (LR-RCC-P-CAPT recipient), which both received components from donor N220.

Discussion

We undertook an independent evaluation to assess the qualitative effects of prion reduction following P-CAPT filtration of leucoreduced red cell concentrates compared to leucoreduced red cell concentrates alone. Unique to this study is that the source components are endogenously infected red cell concentrates from a large animal model. Out of fourteen recipients of LR-RCC or LR-RCC-P-CAPT, we observed infection and clinical disease in two recipients of paired components from the same donor.

Due to the limitations in blood volume that can be collected from an individual sheep at one time, we were unable to carry out transfusions of non-leucoreduced RCC for direct comparison of transmission rates. However, in the related leucodepletion study, similar volumes of RCC prepared from BSE-infected donors at pre-clinical time points (30% - 50% of incubation period) transmitted infection to eight out of twenty-one recipients (38% attack rate), while LR-RCC transmitted to three out of twelve (25%) recipients (unpublished data). There is evidence from previous sheep studies showing that the probability of transmission of BSE or scrapie by blood transfusion increases as donors progress towards the clinical phase of infection,[16,59] probably as a result of increasing titres of infectivity, and that RCC collected from scrapie-infected sheep in the late pre-clinical period can infect 100% of recipients.[42] In support of this, the donor sheep (N220) that transmitted infection in the current study, also provided blood components at a pre-clinical time point (37% of survival period), which failed to transmit infection to their respective recipients (see Table S1).

If we accept that the transmission rate in recipients of non-leucoreduced RCC from clinical donors would be greater than 38% and possibly as high as 100%, the fact that only one out of seven recipients of LR-RCC became infected indicates that leucoreduction alone reduces

the risk of transmission. Since infection was seen in the paired recipient of LR-RCC-P-CAPT from the same donor, it may tentatively be concluded that prion reduction by filtration does not provide an additional benefit to leucoreduction in reducing the risk of transmission. However, as the numbers of animals in the study are too small to give statistical power, we are unable to provide quantitative analysis of the level of risk reduction associated with prion filtration compared to leucoreduction alone. In addition, since we lack a suitable bioassay host (e.g. transgenic mouse) for quantifying BSE infectivity in sheep blood, we cannot provide estimates of titre before and after leucodepletion and prion filtration that would allow calculation of prion clearance values. Our findings are similar to those reported following blood transfusions from scrapie-infected sheep,[42] in which one out of five recipients of leucoreduced red blood cells and one out of five recipients of leucoreduced and prion filtered red blood cells showed evidence of infection when culled at the end of the experiment (but did not develop clinical disease). The latter study tested the effect of the Leukotrap (Pall) and KASEI (Asahi) filters, but not the P-CAPT filter.

At present, the only peer-reviewed data available on the use of the P-CAPT filter relate to its application for removal of exogenous and endogenous infectivity associated with 263K hamster scrapie. Studies using hamster brain homogenate spiked into a full unit of human leucoreduced red blood indicated that processing using the P-CAPT filter resulted in approximately 3 log reduction in infectivity[39]. However, it has been shown that as little as 200 microlitres of intravenously administered blood can transmit infection between scrapie-infected sheep, despite very low infectious titres estimated by intracerebral inoculation of blood into transgenic mice expressing sheep PrP, while the equivalent minimal infectious dose of brain-derived scrapie for sheep by the intravenous route is about 1000 times greater.[20] This implies that results obtained from experiments using exogenous spikes of brain-derived infectivity to measure prion clearance by various processes may not accurately predict effects on endogenous infectivity. Evaluation of the test resins used to develop the P-CAPT filter showed that a combination of leucoreduction and prion filtration reduced

endogenous infectivity in a unit of hamster whole blood by more than 1.22 $\log_{10}$ID[33]. Another recent study, in which 2 units of leucoreduced red cells prepared from scrapie-infected hamsters were filtered using the P-CAPT device, showed an overall reduction in infectivity of approximately 1.4 $\log_{10}$ID, but concluded that there was residual infectivity of around 0.2 ID/ml in the prion filtered blood (written communication; Dr. J.M. Sutton, September 2014).

Leucoreduction of scrapie-infected hamster blood alone has been estimated to remove up to approximately 70% of endogenous infectivity, with the remainder being plasma-associated. However, transfusion experiments using prion-infected sheep and deer suggest that the infectivity in plasma is much lower in these species, and may be largely cell-associated.[17,19,42] The single case of "sub-clinical" vCJD infection identified in a person with haemophilia was thought most likely to have been caused by documented exposure to plasma products derived from a vCJD patient, which suggests that human plasma can also be infectious.[28] These apparent species differences may reflect host-specific variation in the distribution of endogenous infectivity, or may arise from differences in the protocols used for preparation of components in the various studies. In this study, it is possible that the transmission of infection by LR-RCC and LR-RCC-P-CAPT components could be explained by infectivity associated with residual plasma and/or the small numbers of leucocytes remaining following leucoreduction and prion filtration. However, comparable levels of leucoreduction were achieved in components from all seven donors, but only one (N220) transmitted the infection, suggesting that this sheep may have had unusually high titres of blood borne infectivity.

Ten of the sheep that were transfused with either LR-RCC or LR-RCC-P-CAPT are alive, with current survival periods ranging from 1710 to 1984 days post infection (Table 1). There is as yet no clear evidence, from clinical observations and the results of rectal biopsies, that any of these sheep are infected with BSE. The incubation periods of confirmed BSE-infected transfusion recipients from our ongoing leucodepletion study,[17] are 1018 ± 188 days (mean ±

standard deviation) for 141LF genotype sheep (n = 25) and 705 ± 120 days for 141FF sheep (n =15) respectively (unpublished data). For comparison, the majority (9/10) of the surviving recipients in this experiment are 141LF (mean survival period = 1884 ± 118 days post infection), with a single 141FF recipient (survival period = 1773 days post infection). These survival periods represent a difference from the observed incubation periods of more than 4 standard deviations of the mean, suggesting that it is unlikely that the remaining sheep will develop clinical signs. However, it is possible that they will show signs of infection when they are culled, even in the absence of clinical disease. Their prolonged survival after transfusion is consistent with a substantial reduction in infectivity associated with leucoreduction alone or in combination with prion filtration.

In conclusion, we have demonstrated that, for one out of seven BSE-infected sheep used as blood donors, P-CAPT filtration of leucoreduced red cell concentrates did not remove all endogenous infectivity, resulting in infection and clinical disease in the transfused recipient. This supports the view that the filter cannot be assumed to prevent transmission of vCJD by blood transfusion.

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Author contributions

Conceived and designed the experiments: SMC, JM, MLT and other representatives from the SNBTS. Performed the experiments: SMC, EFH, ARAB, BCT, AS, LG, SM, GM and NA. Analysed the data: SMC and ARAB. SMC wrote the publication: All authors contributed to editing and approval of the final version of the report.

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Figure legends

Figure 1: Immunohistochemical labelling of PrP^d to confirm BSE infection. This figure shows a series of representative images taken of the dorsal motor nucleus of the vagus nerve (DMNV, brain), labelled with the PrP monoclonal antibody BG4. Panels A, B and C are from BSE-infected donor sheep N220, N245 and N218, respectively. Panels D, E, F and G are from recipient sheep P511, P467, P548 and P524 respectively. Panel H – positive control; sheep infected with BSE. Panel I – negative control; uninfected sheep. Positive PrP^d labelling, comparable to that of the positive control (panel H), is seen in all three donors (A-C), and in the paired recipients of blood components from donor N220, P511 (panel D) and P524 (panel G). Scale bar = 100 µm.

Figure 2: Western blot detection of PrPSc for confirmation of infection in transfusion recipients. Proteinase K-resistant PrP^{Sc} was prepared from PK digested and NAPTA precipitated tissue homogenates. Tissues from euthanised recipients P524, P511, P467 and P548 were examined. Immunoblots were probed with the antibody ROS-BC6 (0.5µg/ml). Tissues analysed were medulla (pre-diluted 1:10 before loading, lane 1), neat extracts of spleen (lane 2), pre-scapular lymph node (lane 3), mesenteric lymph node (lane 4), distal ileal payer patches (lane 5) and tonsil (lane 6). Molecular weight markers are shown in kDa on the left hand side of the immunoblots and the exposure time was 6 minutes.

Table 1: Details of donor and recipient sheep

Donor details*				Recipient details				
Sheep ID	***PRNP* codon 141 genotype**	**†Survival period (days post infection, dpi)**	**§Component**	**Sheep ID**	***PRNP* codon 141 genotype**	**†Survival period (dpi)**	**Reason for cull**	**Status**
N220	FF	797	LR-RCC	P524	LF	732	Welfare (suspect clinical)	BSE +ve
			LR-RCC-P-CAPT	P511	FF	1049	Clinical	BSE +ve
N218	FF	840	LR-RCC	P548	FF	1130	Welfare	BSE -ve
			LR-RCC-P-CAPT	P466	LF	1984‡	NA	Alive
N245	LF	959	LR-RCC	P467	LF	573	Welfare	BSE -ve
			LR-RCC-P-CAPT	P461	LF	1981‡	NA	Alive
N180	FF	817	LR-RCC	Q391	LF	1970‡	NA	Alive
			LR-RCC-P-CAPT	Q397	LF	1970‡	NA	Alive
N258	LL	944	LR-RCC	P494	LF	1927‡	NA	Alive
			LR-RCC-P-CAPT	P304	LF	1927‡	NA	Alive
N178	LF	1168	LR-RCC	P541	LF	1774‡	NA	Alive
			LR-RCC-P-CAPT	P465	FF	1773‡	NA	Alive
N259	LF	1076	LR-RCC	P473	LF	1710‡	NA	Alive
			LR-RCC-P-CAPT	P280	LF	1710‡	NA	Alive

*All donors were culled, upon reaching defined clinical endpoints associated with BSE infection, immediately after two units of whole blood was collected (i.e. on the day of blood donation).

†Survival periods were calculated as the number of days between the time of infection (by oral route for donors or by transfusion for recipients) and euthanasia OR 3rd October 2014 for sheep that are still alive (indicated by the ‡).

§ LR-RCC = leucoreduced red cell concentrate; LR-RCC-P-CAPT = P-CAPT filtered leucoreduced red cell concentrate.

Table 2: Specifications of blood components transfused to recipients

Component parameters	Donor ID													
	N220		N218		N245		N180		N258		N178		N259	
	U1*	U2†	U1*	U2†	U1*	U2†	U1*	U2†	U1*	U2†	U1*	U2†	U1*	U2†
Volume whole blood (ml)	535	517	502	467§	455§	503	417§	377§	485	490	426§	505	447§	499
Leucofiltration time (minutes)	22	23	18	16	38	22	14	10	19	12	15	19	35	16
P-CAPT filtration time (minutes)	63	-	45	-	70	-	52	-	65	-	56	-	40	-
Volume LR-RCC or LR-RCC-P-CAPT transfused (ml)	289	262	297	284	288	259	251	228	259	258	286	220	247	205§
Leucocyte count (x 10^4 cells/unit)	NA‡	NA‡	14	4.4	50	4.8	1.1	1.3	21	9.8	1.3	1.3	0‖	0‖

*Components prepared from unit 1 (U1) = leucoreduced and P-CAPT-filtered red cells (LR-RCC-P-CAPT).
†Components from unit 2 (U2) = leucoreduced red cells (LR-RCC).
‡NA - Leucocyte count not assessed due to equipment failure on the day these units were processed.
§Components which are lower in volume compared to the specifications for human equivalents. Acceptable volume specifications in human transfusion practice are 468-558ml for a unit of whole blood and 220-340ml for red cell concentrates.
‖ The 0 events recorded for the LR-RCC from donor N259 does not mean that the units contained no leucocytes but rather that the residual numbers were below the limit of detection of the assay,

Table 3: Results of RAMALT biopsy in transfusion recipients

Recipient ID	No. positive follicles / total follicles (% positive)	Sample time (days post infection)
P524	29 / 29 (100%)	676
P511	2 / 10 (20%)	676
	11 / 23 (48%)	763
	2 / 5 (40%)	942
P548	0 / 20	587
	0 / 12	853
P466	0 / 28	587
	0 / 12	853
	0 / 9	1448
P467	NA*	NA*
P461	0 / 24	589
	0 / 36	850
	0 / 4	1445
Q391	0 / 30	573
	0 / 8	839
	0 / 8	1434
Q397	0 / 14	573
	0 / 6	839
	0 / 2	1434
P494	0 / 23	530
	0 / 21	796
	0 / 3	1391
P304	0 / 3	530
	0 / 0	796
	0 / 2	1391
P541	0 / 15	377
	0 / 8	643
	0 / 2	1238
P465	0 / 31	376
	0 / 13	642
	0 / 4	1237
P473	0 / 9	313
	0 / 4	579
	0 / 9	1174
P280	0 / 10	313
	0 / 4	579
	0 / 13	1174

*NA – sheep P467 was euthanized before biopsies were collected.

Figure 1.

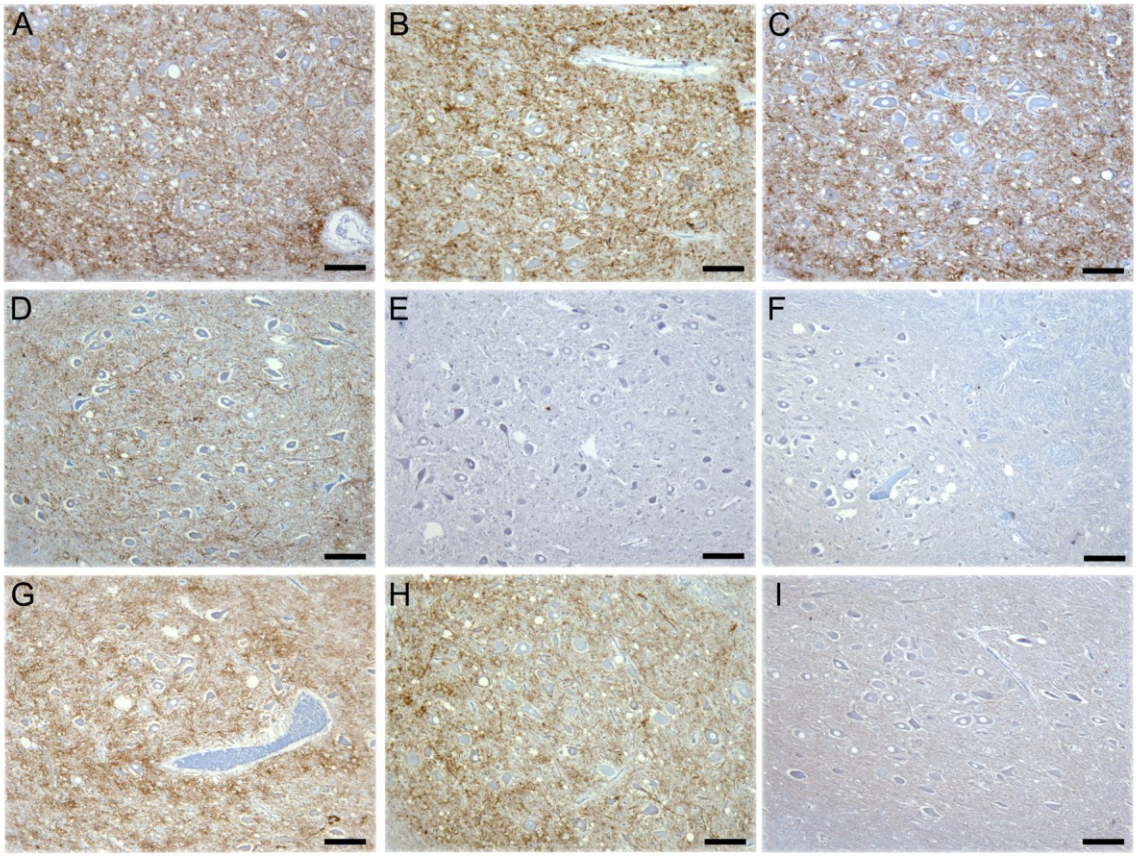

Figure 2.