

# High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrP in vivo

# HIGH TITRES OF TSE INFECTIVITY ASSOCIATED WITH EXTREMELY LOW LEVELS OF $PrP^{Sc}$ *IN VIVO*.

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Running title: Relationship between $PrP^{Sc}$ and infectivity

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**Diagnosis of Transmissible Spongiform Encephalopathy (TSE) disease in humans and ruminants relies on the detection in post mortem brain tissue of the protease resistant form of the host glycoprotein PrP. Presence of this abnormal isoform ($PrP^{Sc}$) in tissues is taken as indicative of the presence of TSE infectivity. Here we demonstrate conclusively that high titres of TSE infectivity can be present in brain tissue of animals which show clinical and vacuolar signs of TSE disease, but contain low or undetectable levels of $PrP^{Sc}$. This work questions the correlation between $PrP^{Sc}$ level and the titre of infectivity, and shows that tissues containing little or no PK resistant PrP can be infectious and harbour high titres of TSE infectivity. Reliance on protease resistant $PrP^{Sc}$ as a sole measure of infectivity may therefore in some instances significantly underestimate biological properties of diagnostic samples, thereby undermining efforts to contain and eradicate TSEs.**

The transmissible spongiform encephalopathy (TSE) diseases (also known as prion diseases) are infectious, fatal neurodegenerative diseases of animals, which include Creutzfeldt Jacob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. The true identity of the infectious agent responsible for these diseases is not known. However, it has been proposed that TSE disease is caused by an abnormal form of the host glycoprotein, PrP (1). The abnormal, disease associated form of the protein ($PrP^{Sc}$), is partially protease resistant and detergent insoluble unlike the normal cellular conformer ($PrP^{C}$), and is seen to accumulate in diseased tissues. The prion hypothesis predicts that $PrP^{Sc}$ alone is the infectious agent of TSE, and is able to induce the conversion of endogenous $PrP^{C}$ into the abnormal form during disease (2).

Most human TSE diseases are familial or sporadic, but disease can also be acquired by surgical intervention (3) or blood transfusion from infected individuals (4-9), or possibly from the consumption of BSE infected meat products, the presumed cause of variant CJD (vCJD) (10). The extent to which vCJD infection in particular is present in the UK population is unknown, but recent research has suggested there may be a higher rate of sub-clinical or pre-clinical vCJD than previously thought in different human PrP genotypes (7,11-13). Although BSE is declining in the UK, cases have now been observed in cattle in countries that have not previously reported BSE. It is also unknown whether the agent responsible for BSE has re-entered the human food chain following transmission to sheep. For these reasons a high level of active and passive surveillance of ruminants is required at slaughter to monitor and prevent TSE infected material from entering the human food chain. The introduction of ante-mortem surveillance in the human population is also critical to prevent the human-to-human transmission of vCJD by blood transfusion or surgical procedures. This will be of particular importance if subclinical disease proves to be

a significant risk in vCJD transmission (12,13).

Positive identification of TSE infectivity can only be demonstrated conclusively by transmission of disease to laboratory animals. Such assays are time consuming, due to long incubation times, and expensive, and are therefore not suitable for the rapid diagnosis of all ante- or post-mortem samples. Current diagnostic tests instead rely on the detection of disease associated $PrP^{Sc}$ in samples taken from brain post-mortem. The development of ante-mortem diagnostic tests is also being based around more sensitive assays for $PrP^{Sc}$. Several diagnostic tests are available commercially, and most require proteinase K (PK) treatment of tissue homogenates to isolate disease specific PK resistant $PrP^{Sc}$ (PrP-res). It has not yet been definitively proven that $PrP^{Sc}$ is the TSE infectious agent, and whether it is present in all infected tissues. Studies using 263K hamster scrapie have shown a strong correlation between PrP-res and infectivity (2,14,15). However other studies have demonstrated the transmission of disease from infected animals which appear to lack significant levels of PrP-res (16-19). In such cases it has been suggested that a PK-sensitive form of $PrP^{Sc}$ ($sPrP^{Sc}$) may represent the infectious agent (20-22). Hence it is possible that infectivity may be associated with a specific isoform of abnormal PrP. The identification of this specific conformer is imperative for the future of TSE diagnosis. However, if TSE infectivity does not always associate with high levels of $PrP^{Sc}$, current diagnostic methods may fail to identify all animals with TSE disease, and may not provide a realistic estimate of the level of infectivity in an infected tissue. For the purposes of this study, $PrP^{Sc}$ is used to define all abnormal forms of PrP, whereas PrP-res specifically defines PK-resistant PrP, and $sPrP^{Sc}$ defines PK-sensitive forms of $PrP^{Sc}$. If present, large amounts of $PrP^{Sc}$ may be a clear indication of the presence of infectivity in a tissue sample.

We have previously identified two mouse models of TSE disease (18,19) which indicate that the association between PrP-res and infectivity is not as straightforward as predicted by the prion hypothesis. Unlike wild-type controls, transgenic mice homozygous for a targeted mutation at amino acid 101 (proline to leucine) in endogenous murine PrP (101LL) develop clinical TSE disease following inoculation with hamster 263K scrapie or human Gerstmann Sträussler Scheinker (GSS) P102L disease (patient shown to contain vacuolar pathology and PrP-res at post mortem) (18,19). Pathological analysis of brain tissue from these mice (101LL/GSS and 101LL/263K) showed TSE associated vacuolation, and the disease could be further transmitted to 101LL mice with short incubation times of 100-160 days (18,19). Such incubation times were indicative of a high titre of infectivity in the 101LL/GSS and 101LL/263K tissues, yet analysis by immunoblot revealed that most animals contained extremely low levels of PrP-res, and several contained no detectable PrP-res at all (18,19). However, the presence of high titres of infectivity cannot be proven by a short disease incubation time. In order to establish the true relationship between $PrP^{Sc}$ and infectivity we have now performed more detailed and quantitative analyses of the disease in these mice. The $ID_{50}$ (dilution at which 50% of the animals become infected) and titre of infectivity in several 101LL/GSS and 101LL/263K infected brains have been established by bioassay. Corresponding levels of PrP-res in the same tissues have also been established semi-quantitatively by immunoblot. These analyses have shown no relationship between infectivity titre and PrP-res level. Moreover no other disease-associated forms of PrP were detectable in these tissues. Thus within our model system there is a clear dissociation between titre of infectivity and level of $PrP^{Sc}$.

## Experimental procedures.

*Transgenic mouse lines and tissues* - Inbred gene targeted transgenic mouse line 101LL, and the corresponding inbred 129/Ola wild type control line have been described previously (18). 101LL/GSS tissues were

produced by inoculation of 101LL transgenic mice with 1% brain homogenate prepared from the occipital cortex of a GSS P102L brain showing numerous multicentric plaques and abundant PrP-res by immunoblot. The individual was 129 methionine homozygous with a confirmed proline to leucine mutation at codon 102 (J. W. Ironside and M. W. Head, personal communication). 101LL/263K tissues were produced by inoculation of 101LL transgenic mice with 1% brain homogenate from a 263K infected hamster. Control tissues were produced by ME7 inoculation of 129/Ola wild type mice and 101LL transgenic mice.

*Preparation of inocula* - Separate inocula were prepared from the brains of two 101LL/GSS and three 101LL/263K infected mice with terminal TSE disease which had been shown by immunohistochemical (IHC) analysis to contain extremely low levels of PrP deposition. Inocula were also prepared from brains of one wild type and one 101LL mouse with terminal ME7 scrapie as controls. A 10% homogenate of each sample was prepared in sterile saline prior to use as an inoculum. This inoculum was then used to produce a series of 10-fold dilutions from $10^{-2}$ to $10^{-9}$ in sterile saline. Each dilution (20µl) was inoculated intracerebrally under anaesthesia into groups of 101LL mice for 101LL/ME7, 101LL/GSS and 101LL/263K tissues, or wild type 129/Ola mice for 101PP/ME7 tissue. All experimental protocols were submitted to the Local Ethical Review Committee for approval before mice were inoculated. All experiments were performed under licence and in accordance with the UK Home Office Regulations (Animals (Scientific Procedures) Act 1986).

*Scoring of clinical TSE disease* - The presence of clinical TSE disease was assessed as described previously (23). Animals were scored for clinical disease without reference to the genotype of the mouse. Genotypes were confirmed for each animal by PCR analysis of tail DNA at the end of the experiment. Incubation times were calculated as the interval between inoculation and cull due to terminal TSE disease. Mice were killed by cervical dislocation at the terminal stage of disease, at termination of the experiment (between 500-700 days), or for welfare reasons due to intercurrent illness. The proportion of mice showing positive vacuolar pathology was calculated for each group, and the $ID_{50}$ (dilution at which 50% of the mice became infected) was determined using the Karber method (24). This value was used to calculate the number of infectious units per gram wet weight of tissue (IU/g).

*Genotyping of mouse-tail DNA* - A 2-3cm portion of tail was removed post-mortem from each mouse. DNA was prepared and the PrP genotype of each mouse confirmed as described previously (18).

*Immunoblot analysis and quantification of PrP-res* - For immunoblot analysis, residual inocula (10% saline homogenate) were mixed with an equal volume of 2x NP40 buffer [2% NP40, 1% sodium deoxycholate, 300mM NaCl, 100mM Tris/HCl pH7.5] and further homogenised in a microcentrifuge tube using 20-30 strokes with a pre-cooled centrifuge tube pestle (Anachem). The homogenate was centrifuged at 11,000g for 10min at $10^0$C to remove cellular debris, and the supernatant stored in 50µl aliquots at -$70^0$C. For quantification of PrP-res levels in each tissue, homogenates were digested with 20µg/ml proteinase K (PK) at $37^0$C for 1h. Digested homogenates were diluted to 1%, and two-fold serial dilutions were prepared using PK treated normal brain homogenate as the diluent to keep overall protein concentrations constant. Diluted samples were mixed with sample loading buffer and sample reducing agent (Invitrogen) and loaded across two 12% tris/glycine polyacrylamide gels (Invitrogen) at concentrations ranging from 1mg/ml to 3.9µg/ml (200µg to 0.8µg wet weight tissue equivalent). 50ng of recombinant PrP was loaded onto each gel as an internal control. After separation, proteins were transferred onto PVDF membrane by electroblotting and PrP was detected with monoclonal antibody 8H4 (West Dura ECL substrate; Pierce). Monoclonal antibody 7A12 and polyclonal antibody 1B3 were also used to confirm the low PrP-res levels in 101LL/GSS and 101LL/263K tissues. Images were captured on both X-ray film and by a Kodak Digital

Image Station 440. Experiments were repeated in duplicate or triplicate depending on sample availability.

Digital images of each gel were analysed using Kodak ID software, and PrP-res levels expressed as pixel intensities. Samples were normalised across the two blots and quantified using the recombinant PrP controls as standards. Each value was multiplied by the dilution factor and an average was taken for all samples run per tissue to determine the level of PrP-res per gram wet weight brain tissue in each model. This value, combined with the titre of TSE infectivity measured in each tissue (IU/g) was used to calculate the number of molecules of PrP-res per infectious unit for each tissue as follows;

*Number PrP-res Molecules per gram of tissue = n*

*n = [PrP-res per gram (g) / Avagadro's number ($6.02 \times 10^{23}$)] / Mwt PrP (30,000)*

*Number of molecules PrP-res per infectious Unit = n / titre (IU/g)*

*Measurement of alternative forms of PrP* - The proteinase K (PK) resistance of PrP in all samples was analysed by digestion with a range of PK concentrations. Individual 9µl aliquots of each 5% NP40 brain homogenate were incubated at $37^0$C for 1h with PK concentrations ranging from 1µg/ml to 20µg/ml. The reaction was terminated by addition of Phenylmethylsulphonyl fluoride (PMSF) to 1mM, and samples were analysed by SDS PAGE and immunoblotting as described above.

For "cold PK" digestion, samples (10% homogenate) were incubated with 250µg/ml PK on ice for 1h. Digestion was stopped by the addition of PMSF to 1mM. Samples were de-glycosylated with PNGase F (New England Biolabs) following the manufacturers instructions, and analysed by SDS PAGE and immunoblotting.

*DELFIA analysis* - Dissociation Enhanced Lanthanide Fluoro Immuno Assay (DELFIA) analysis of brain homogenate was performed using a modification of the method described by Barnard *et al.* (25). Briefly, 50µl of 10% PBS brain homogenate was mixed with an equal volume of 2M GdnHCl and homogenised in a microfuge tube with a microfuge pestle (Anachem). Samples were re-homogenised after addition of 50µl of 1x NP40 buffer, vortexed for 2 min and mixed with 850µl of assay buffer (Perkin Elmer). Samples were centrifuged at 13,000g for 10 min to produce Supernatant 1 (1M GdnHCl soluble fraction). Pellets were re-suspended in 100µl of 6M GdnHCl, mixed with 900µl of assay buffer and spun at 13,000g for 10 min to produce Supernatant 2 (1M GdnHCl insoluble fraction). 1M GdnHCl soluble and insoluble fractions were added to 96 well plates pre-coated with MAb FH11, and PrP levels in each fraction were detected with Europium labelled MAb 7A12 according to the manufacturers' instructions (Perkin Elmer). Recombinant PrP standards were included in each assay for calibration. Samples were assayed in duplicate, and the percentage coefficient of variation calculated for each sample pair.

*Immunoprecipitation of $PrP^{Sc}$.*

Laterally bisected brain halves from 101LL transgenic mice were homogenised at 10% (w/v) in Tris-buffered saline (TBS) and diluted to reach a concentration of 5% (w/v) in TBS containing 1% Triton. Homogenates were sonicated for three pulses of 4 seconds and clarified by centrifugation at 400g for 10 min at $4^o$C. PMSF was added to all samples to a concentration of 2mM. Each sample was analysed by dot blot to estimate the total PrP content. Briefly, brain homogenates were serially diluted (1:1) in TBS containing 1% Triton then denatured in Tris-SDS sample buffer at $100^o$C for 5 min. Equivalent amounts of each sample were then deposited on a nitrocellulose membrane and left until dry. The membrane was probed with Mab 6H4 (Prionics) and an HRP labelled anti-mouse secondary antibody (Pierce). The resulting signals were compared semi-quantitatively. These data were used to ensure equal PrP input into each individual immunoprecipitation (IP) reaction. For each IP reaction, the motif grafted antibodies or control antibodies were incubated at 10µg/ml final concentration for 2h at RT in a reaction mixture including 1% Triton. Rabbit anti-human antibodies (Jackson) coupled to magnetic dynabeads (Dynal) were used to

capture the PrP-specific antibodies as described (26,27). Immunoblot membranes were probed with Mab 6H4 and developed using the ECL femtomolar kit (Pierce).

## RESULTS.

**101LL mice infected with 263K and GSS P102L show little PrP deposition in brain;** Brain tissue from 101LL transgenic mice which showed TSE clinical signs and TSE associated vacuolar pathology following inoculation with hamster 263K scrapie or human GSS P102L (18,19) was screened for PrP deposition by immuno-histochemistry (IHC) using anti-PrP monoclonal antibody 6H4. As previously demonstrated, 101LL/GSS and 101LL/263K infected mice had low levels of PrP deposition in the brain, despite having confirmed TSE disease. Three 101LL/263K and two 101LL/GSS infected tissues, which showed extremely low PrP deposition in the brain, were selected for further analysis by bioassay (Figure 1 and Table 1). In each case, PrP deposition was restricted to the thalamus, and in most cases was only visible as small grainy deposits under high power microscopy (Figure 1G-I). Low or undetectable levels of PrP-res in each brain homogenate were confirmed by immunoblot following PK treatment of residual inoculum (Figure 2).

**High levels of infectivity can be measured by bio-assay of 101LL/GSS and 101LL/263K brain tissue.** Although short incubation times in mice can be indicative of high levels of TSE infectivity in an inoculum, the actual level can only be determined by establishing the $ID_{50}$ (dilution at which 50% of the animals become infected) for the inoculum. Infectivity titres were therefore established for the five selected tissues; 101LL/263K(a), 101LL/263K(b), 101LL/263K(c), 101LL/GSS(d) and 101LL/GSS(e) (Table 1). It was considered extremely important in these experiments that, as far as possible, a single brain be used for each series of procedures ($ID_{50}$ determination, PK digestion, IHC, etc..). This allowed direct correlation to be made between the level of infectivity and PrP-res in each individual brain and avoided any variation which may occur between tissues, as is often observed on a primary transmission. Moreover this approach avoided the necessity of carrying out large numbers of titration experiments which would have been both impractical and ethically unacceptable. Inocula were prepared from each individual tissue as 10% sterile saline homogenates, and used to produce a series of 10-fold dilutions ($10^{-2}$ to $10^{-9}$) for inoculation. Wild type control 129/Ola and transgenic 101LL mouse brains infected with the well characterised mouse scrapie strain ME7 (Wt/ME7 and 101LL/ME7 respectively) (18) were also assayed as controls. The seven samples were inoculated intracerebrally (i.c.) into groups of 129/Ola mice for Wt/ME7, and transgenic 101LL mice for all other samples. The percentage of mice which developed TSE pathology was calculated for each group in each dilution series, and the $ID_{50}$ was determined using the Karber calculation (24). The number of infectious units per gram tissue (IU/g) for each individual mouse brain are shown in Table 2. Assuming a ± 0.5 log error for each titre (24), all 101LL/GSS and 101LL/263K samples produced titres of infectivity ranging from ~$10^7$ to $10^9$ IU/g. The highest titre ($10^{9.8}$) was identified in 101LL/GSS(d), however a titre of $10^{8.7}$ was also identified in 101LL/263K(a). Both of these brains showed low levels of PrP deposition by IHC, but titres were higher than that measured in control Wt/ME7 brain ($10^{8.5}$) which showed significantly more PrP deposition by IHC (Figure 1). Titres in the other three tissues were similar ($10^{7.2}$ to $10^{7.5}$) and confirmed a high level of infectivity in the presence of extremely low or undetectable PrP deposition in the brain (Figure 1 and 2). The results of the $ID_{50}$ determination therefore prove the presence of high levels of infectivity in 101LL transgenic mice infected with P102L GSS or hamster 263K.

**Little or no PrP-res is detected in highly infectious tissue.** IHC using anti-PrP monoclonal and polyclonal antibodies found little or no PrP deposition in brain tissue of 101LL/263K and 101LL/GSS infected mice (Figure 1, and data not shown). However, IHC does not distinguish between different forms

of PrP, therefore direct measurement of brain PrP-res levels was undertaken to determine the amount of PrP-res associated with titre of infectivity in each brain, listed in Table 1. Residual inoculum from each bioassay was mixed with detergent buffer, digested with PK (Figure 2) and a 2-fold serial dilution from 1mg/ml to 3.9µg/ml (wet weight brain tissue) analysed by immunoblotting with monoclonal antibody 8H4 (28). Recombinant PrP (rec-PrP) was loaded on each gel at 50ng as an internal control. For the ME7 infected tissues, the limit of PrP-res detection was 15.6µg/ml for Wt/ME7 homogenate, and 31.3µg/ml for 101LL/ME7 homogenate. Hence the same agent produced approximately 2-fold less PrP-res in the 101LL transgenic mice compared to wild type mice, although this was associated with a 0.7 log drop in titre (Table 2). In 101LL/263K(a) the limit of PrP-res detection was 62.5µg/ml brain homogenate, which was approximately half the level in 101LL/ME7 and one quarter the level in Wt/ME7. For all other samples, no PrP-res was detectable in even the most concentrated (1mg/ml) sample examined (Table 2, Figure 2, Supplemental Figure 1A). Digital imaging of immunoblots and quantitation of PrP-res relative to recombinant PrP control, allowed the calculation of $PrP^{Sc}$ concentration (mean PrP-res gram per gram wet weight of tissue) in each sample (Table 2). The level of sensitivity for the immunoblot, determined using recombinant PrP, was 5-10ng, therefore the level of PrP-res in samples which showed no PK-resistant material must be below this threshold. Previous studies have shown that PrP-res from other well characterised rodent scrapie strains with titres ranging from $10^{5.5}$ to $10^{9}$ can be easily identified on immunoblot of 1% brain homogenate following PK treatment (Supplemental Figure 1B). These data would suggest that tissue containing titres of $10^{7}$ to $10^{9}$ IU/g should contain levels of PrP-res which can be easily identified by immunoblot. However for 101LL/GSS and 101LL/263K infected tissue this is clearly not the case. While we cannot eliminate the possibility that PrP-res was indeed present below the threshold level of the immunoblot, a poor correlation between the level of infectivity and the amount of PrP-res in the brain is nevertheless clearly established. In order to confirm that the failure to detect PrP-res on these immunoblots was not simply a consequence of the loss of the monoclonal antibody epitope (8H4) duplicate blots were also probed with a second monoclonal antibody (7A12) and a polyclonal antibody (1B3) which detects multiple epitopes in PrP. These results confirmed the low PrP-res levels in 101LL/GSS and 101LL/263K tissues (data not shown). While the combination of monoclonal and polyclonal antibodies used to examine these tissues make it unlikely that a form of PrP-res exists which has not been detected in our immuno-assays, this possibility has not been totally excluded and we continue to investigate these tissues with new antibodies.

**Are alternative forms of PrP associated with infectivity?** Although PrP-res was present at low or undetectable levels in tissues from 101LL/GSS and 101LL/263K infected mice, it is possible that forms of PrP other than PrP-res may be infectious (29). Alternative forms of PrP such as transmembrane PrP (30,31) cytoplasmic PrP (32,33), and PrP with amino acid insertions or deletions (34-37) have been linked with disease. In addition, a PK-sensitive variant of $PrP^{Sc}$ ($sPrP^{Sc}$), has been recently described (20-22), which may represent an intermediate in the refolding of $PrP^{C}$ to $PrP^{Sc}$ during the disease process, and could therefore be associated with infectivity. In order to test whether $sPrP^{Sc}$ may account for the dissociation between PrP-res and infectivity in 101LL/263K and 101LL/GSS tissues we examined the protease resistance of PrP in such brains by digesting with a range of PK concentrations from 1µg/ml to 20µg/ml. Homogenates from Wt/ME7, 101LL/ME7 and uninfected 101LL and 129/Ola mice were also treated with varying PK concentrations as controls. Digestion was stopped by the addition of PMSF to 1mM, and samples were analysed by immunoblot (Figure 3). In the positive controls (Wt/ME7 and 101LL/ME7) PrP-res was evident in all dilutions, with the PK-resistant core still visible after treatment with 20µg/ml PK (data not shown). PrP in the

uninfected controls was found to be sensitive to PK concentrations greater than 5µg/ml, and produced mildly PK-resistant fragments at PK concentrations of 2-5µg/ml under the digestion conditions used here (Figure 3). PrP in the 263K infected 101LL brains showed variable PK resistance, in agreement with the level of PrP-res detectable in each homogenate. Thus, 101LL/263K(a) showed PrP-res at 20µg/ml, but 101LL/263K(b) & (c) showed a similar pattern of PK-resistance to uninfected mice. In addition, samples from both 101LL/GSS(d) (Figure 3) and 101LL/GSS(e) showed a PK sensitivity pattern identical to that of uninfected 101LL mice.

The presence of $sPrP^{Sc}$ in brain tissue has also been demonstrated by performing "cold PK digestion", i.e. PK digestion on ice (21,22). $sPrP^{Sc}$ has been previously identified in samples which showed no PrP-res (using standard digestion conditions of 20µg/ml for 1h at $37^0$C) by the presence of a 22kDa band on immunoblot after digestion with PK on ice and subsequent de-glycosylation with PNGase F (21,22). Although we aimed to perform all procedures on each individual mouse brain, the limited tissue size meant this was not possible for the "cold PK" analyses carried out here. However "cold PK" digestion was performed on brain tissue taken from mice showing positive clinical and vacuolar signs of TSE, but low levels of PrP deposition in the same primary transmission experiments as those listed in Table 2 (details in Figure S2 and Table S1). These tissues failed to demonstrate any marked increase in the 22kDa PK-resistant PrP band after "cold PK" digestion (Figure 4, lanes 2 and 3). When compared to the ME7 control (Figure 4 lane 6, loaded at 25% concentration of lanes 2-5), the low levels of PrP apparent in lanes 2 and 3 after digestion with PK on ice demonstrate that $sPrP^{Sc}$ cannot account for the high titre of infectivity in the 101LL/263K and 101LL/GSS models.

Although $PrP^{Sc}$ is generally defined by its partial resistance to PK digestion, it can also be identified by its differential solubility in guanidine hydrochloride (Gdn-HCl). While $PrP^{C}$ is soluble in 1M Gdn-HCl, $PrP^{Sc}$ is not, and the two forms can therefore be separated by centrifugation of brain homogenate in 1M Gdn-HCl. Resulting pellets can be solubilised in 6M Gdn-HCl and the two fractions (1M and 6M) assayed for PrP content by DELFIA sandwich ELISA (25). Samples from 101LL/GSS(d) and 101LL/GSS(e) infected mice which contained no PrP-res by immunoblot but showed titres of $10^{9.8}$ and $10^{7.2}$ IU/g by bioassay were subjected to this analysis. PrP levels detected in the 1M Gdn-HCl insoluble fraction by DELFIA were similar to those in uninfected mice (Figure 5), indicating that no $PrP^{Sc}$-like conformers were present in these tissues. Levels of PrP detected in the 1M soluble fraction were variable, but fell within the range observed for control (uninfected) 101LL mice, indicating that PrP levels in brain tissue harbouring titres of $10^{7.2}$-$10^{9.8}$ IU/g could not be differentiated by PrP-specific DELFIA from those in uninfected control 101LL mouse tissue. 101LL/263K samples which had been titrated by bioassay were not analysed by DELFIA due to limited sample availability. However one further brain from the same 263K transmission experiment which showed clinical and vacuolar signs of TSE but did not contain PrP-res by immunoblot was analysed and found to contain no 1M Gdn-HCl insoluble material (Figure 5).

**Immunoprecipitation using $PrP^{Sc}$ specific monoclonal antibodies.** Several monoclonal antibodies (MAbs) have been generated which specifically bind $PrP^{Sc}$ isoforms, but not $PrP^{C}$. These antibodies can therefore isolate $PrP^{Sc}$ from non-PK treated tissue homogenates by immunoprecipitation, ensuring that all abnormal PrP isoforms are identified. This technique has been used by others to demonstrate the presence of $sPrP^{Sc}$ in the brains of mice overexpressing 101L-PrP (22). Here, $PrP^{Sc}$-specific motif grafted MAbs 89-112 and 136-158 (26) were used to immunoprecipitate PrP from brain tissue homogenates of 101LL/GSS and 101LL/263K infected mice. Tissues analysed were taken from mice showing positive clinical and vacuolar signs of TSE, but low levels of PrP deposition in the same primary transmission experiments as those used to determine titre of

infectivity in each model (details in Figure S2 and Table S1). Positive control MAb D13 (which precipitates only the cellular form of PrP) and negative control MAb b12 were also included in all experiments. For all 101LL/GSS and 101LL/263K tissues examined, extremely low levels of $PrP^{Sc}$ were immunoprecipited by both $PrP^{Sc}$-specific antibodies (Figure 6). These levels were estimated by immunoblot to be 100-1000 fold less than those precipitated from control RML infected mouse brain. Results from these immunoprecipitations therefore support our previous biochemical data which show no evidence of PK-sensitive forms of $PrP^{Sc}$ in brain tissue from 101LL/GSS and 101LL/263K infected mice.

## DISCUSSION.

$PrP^{Sc}$ is thought to be the sole component of the prion, or TSE infectious agent. For this reason it has become the main target for TSE diagnostic assays, where identification of $PrP^{Sc}$ in post-mortem brain tissue indicates a TSE positive animal. However the relationship between $PrP^{Sc}$ and TSE infectivity has not been definitively demonstrated, and concerns have been raised by earlier reports of disease transmission in the apparent absence of PrP-res (16,18). In particular, 101LL gene targeted transgenic mice inoculated with GSS P102L or 263K succumb to a disease which is highly transmissible to both 101LL and wild type mice, but shows extremely low levels of PrP-res in the brain. Extended analyses of this model (described here) have now used quantitative assays to unequivocally demonstrate that titres of $10^7$ to $10^9$ IU/g can be present in brain tissue which shows little or no abnormal PrP accumulation by standard immunoblot analysis, IHC, DELFIA or immunoprecipitation. These titres are similar to or higher than those observed in our well characterised, high titre control strain ME7, but for 4 of the 5 brains analysed, PrP-res levels were below the limit of detection of our immunoblot assay (<1.3% of the amount of PrP-res in wild type ME7 tissue). Previous studies have shown that PrP-res from other well characterised rodent scrapie strains with titres ranging from $10^{5.5}$ to $10^9$ can be easily identified on immunoblot of 1% brain homogenate following PK treatment. Based on these previous data, it would be predicted that the tissues studied here should contain titres far below $10^5$ IU/g tissue. However the transmission data clearly show that 101LL/GSS and 101LL/263K infected tissues contained high titres of infectivity which exceed those measured in both 79V and 22A infected tissue (Figure S1B). These data suggest that current diagnostic assay systems which rely on $PrP^{Sc}$ detection might fail to identify some highly infectious tissues. To this end, tissues from 101LL/GSS and 101LL/263K infected mice are currently being assessed in several of these assay systems in our laboratory.

Several independent studies have previously shown that one TSE infectious unit is composed of approximately $10^5$ $PrP^{Sc}$ molecules (2,14,15). In contrast to these studies the data obtained from 101LL/GSS and 101LL/263K infected tissues indicate that the number of $PrP^{Sc}$ molecules per unit of infectivity must display a wide range, with 101LL/GSS and 101LL/263K tissues showing between 10 to 1000 times fewer PrP-res molecules per unit infectivity than Wt/ME7. Alternatively, these data could indicate that only a very small proportion of $PrP^{Sc}$ present in TSE infected tissue is actually infectious. This lack of correlation between levels of PrP-res and infectivity do not support PrP-res as the infectious agent of TSE.

As PrP-res does not appear to be a major component of infectivity in this study, it is possible that another form of PrP is responsible for disease in these mice. We have shown previously that 101LL mice can form PrP-res when inoculated with other rodent TSE strains (38), therefore the lack of $PrP^{Sc}$ in these models is not due to an inherent inability of 101L-PrP to convert to a protease resistant isoform. In contrast to the gene targeted transgenic 101LL mice described here, transgenic mice which overexpress 101L-PrP at levels 8-16 fold higher than endogenous PrP develop a spontaneous neurological disease which appears to be

associated with a PK-sensitive form of $PrP^{Sc}$ (21,22). We have found no evidence of $sPrP^{Sc}$ in 101LL/GSS or 101LL/263K brain tissue by either limited PK digestion studies or differential extraction in GdnHCl. Additionally, motif grafted MAbs which specifically bind $PrP^{Sc}$ did not immunoprecipitate PK-sensitive forms of $PrP^{Sc}$, as had been observed in sick 101L overexpressing transgenic mice. One possible reason for this discrepancy between models is that disease in 101LL/GSS and 101LL/263K mice is due to a TSE infection which has been transmitted from a known infected source, and can be further passaged to both 101LL and wild type 129/Ola mice (18,19). In contrast the disease observed in transgenic mice overexpressing 101L PrP does not transmit to wild type mice, and only appears to accelerate the phenotype already present in mice expressing lower levels of the transgene (17,22), suggesting that $sPrP^{Sc}$ may instead be associated with overexpression of 101L-PrP and not TSE disease. The species of abnormal PrP produced due to overexpression of 101L-PrP is therefore different from that produced by TSE infection. The nature of the infectious agent in the current study has yet to be established. We now aim to use this unique model to determine whether infectivity in these tissues is consistent with other abnormal conformations of PrP or with factors other than PrP.

The models of disease described here demonstrate the potential for the existence of high levels of TSE infectivity with undetectable PrP-res in natural disease. Indeed, increased surveillance and sensitivity of testing methods has identified a new TSE of sheep, termed atypical scrapie. These animals were identified as TSE infected by one $PrP^{Sc}$ specific diagnostic ELISA, but could not be confirmed by other methods (39,40). Such cases are now only identifiable using assays which require low concentrations of PK, or no PK in the assay procedure. It is unknown whether this is truly a new TSE of sheep, or whether it has been present in sheep for some time, but was not detected due to the reduced PK-resistance of $PrP^{Sc}$. However the disease has been shown to be highly transmissible to transgenic mice expressing ovine PrP (41) indicating the presence of substantial levels of infectivity. The results of our study raise concern over the suitability of $PrP^{Sc}$ as a sole diagnostic marker of TSE disease. It is vital that markers of TSE infectivity other than $PrP^{Sc}$ are identified and validated in models such as those we have described and characterised here. We anticipate that such research will lead to the development of more robust diagnostic assays for TSE disease which will have important implications for both animal and human health.

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## FOOTNOTES

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Abbreviations used: TSE, Transmissible Spongiform Encephalopathy; CJD, Creutzfeldt Jacob disease; vCJD, variant Creutzfeldt Jacob disease; PK, proteinase K; GSS, Gerstmann Sträussler Scheinker; DELFIA, Dissociation Enhanced Lanthanide Fluoro Immuno Assay; IP, immunoprecipitation; IHC, immunohistochemistry; MAbs, monoclonal antibodies.

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Abbreviations: TSE, Transmissible Spongiform Encephalopathy; vCJD, variant CJD; PK, proteinase K; GSS, Gerstmann Sträussler Scheinker; Wt, wild type; PrP-res, PK resistant PrP; $sPrP^{Sc}$, PK-sensitive $PrP^{Sc}$.

## FIGURE LEGENDS.

**Figure 1. Low levels of PrP deposition in 101LL/GSS and 101LL/263K infected brain.** Immunohistochemistry was performed on sections of brain from 101LL/263K and 101LL/GSS infected mice using monoclonal antibody 6H4 to determine the levels of PrP deposition. ME7 infected control mouse brain was stained as control (F). Five brains (3 x 101LL/263K and 2 x 101LL/GSS) showing very low levels of deposition were selected for further analysis to quantify the levels of TSE infectivity and $PrP^{Sc}$ in each tissue. Very low levels of PrP deposition were observed in brain tissue, which varied between each individual mouse brain. Deposition was mainly observed in the thalamus (Frames G-I). Frames A-F at x4 magnification, Frames G-I at x20 magnification.
**A:** 101LL/263K(a). **B:** 101LL/263K(b). **C:** 101LL/263K(c). **D:** 101LL/GSS(d). **E:** 101LL/GSS(e). **F:** WT/ME7 control. **G:** thalamus of 101LL/263K(a). **H:** thalamus of 101LL/263K(c). **I:** thalamus of 101LL/GSS(d)

**Figure 2. Low or undetectable levels of PrP-res in 101LL/GSS and 101LL/263K infected brain.** Residual inoculum from the tissues selected for $ID_{50}$ bioassay were analysed by immunoblot following PK treatment to detect PrP-res. Lanes 2 ,4, 6, 8, 10 and 12 digested with PK at 20µg/ml for 1h at $37^0$C. Lanes 1, 3, 5, 7, 9 and 11 no PK control. Lanes 1 and 2, uninfected Wt 129/Ola mouse; Lanes 3 and 4, 101LL/263K(b); Lanes 5 and 6, 101LL/263K(c); Lanes 7 and 8, 101LL/263K(a); Lanes 9 and 10, 101LL/GSS(d); Lanes 11 and 12, 101LL/GSS(e). Blots probed with Mab 8H4.

**Figure 3. PK resistance of PrP in 101LL/GSS and 101LL/263K brain tissue.**
Brain homogenates in NP40 lysis buffer were digested with varying concentrations of proteinase K at $37^0$C for 1h. Samples were subjected to SDS PAGE and immunoblotting to determine the PK sensitivity of the PrP present in 101LL/GSS and 101LL/263K tissue. Representative images showing; **A**, uninfected 101LL control mouse brain; **B**, uninfected Wt 129/Ola control mouse brain; **C**, 101LL/263K(a) mouse brain; **D**, 101LL/GSS (d) mouse brain; **E**, 101LL/263K(b) mouse brain. PK concentration used for digestion shown above each lane (μg/ml). Blots probed with MAbs 8H4 (B, C and E) or 7A12 (A and D). Bars indicate molecular weight markers of 36kDa and 30kDa.

**Figure 4. Cold PK Treatment of tissues from high titre/low PrP-res models.**
101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to "Cold PK" digestion on ice. Uninfected and Wt/ME7 infected brains were also digested as controls. Lane 1, undigested 101LL/GSS brain homogenate; Lane 2, 101LL/263K(g); Lane 3, 101LL/GSS(f); Lane 4, 101LL uninfected control; Lane 5, Wt 129/Ola uninfected control; Lane 6, Wt/ME7 infected control. Lanes 2-6 were treated with 250μg/ml PK on ice for 1h and de-glycosylated with PNGase F. ME7 control was loaded at approximately 25% of the concentration of lanes 2-5 to allow comparison. Blot probed with MAb 7A12. Image has been cropped from a single blot to remove lanes with samples which are not relevant to this figure.

**Figure 5. DELFIA analysis of 101LL/GSS brain homogenate.**
Samples of 101LL/GSS brain homogenate, 101LL/263K homogenate and uninfected or ME7 infected controls were analysed for presence of 1M GdnHCl insoluble PrP by DELFIA. Light bars represent readings obtained from 1M GdnHCl soluble fractions, dark bars represent 1M GdnHCl insoluble fractions. Sample 1, 101LL/GSS(d); Sample 2, 101LL/GSS; Sample 3, 101LL/263K; Sample 4, uninfected 101LL Tg mouse; Sample 5, ME7 infected 129/Ola mouse. Sample 2 was a further tissue from the same experiment as 101LL/GSS(d) which showed no detectable PrP-res by immunoblot, but produced short incubation times on subpass to 101LL mice. Due to the limitations of using a single mouse brain for each analysis, insufficient material was available to perform DELFIA analysis on 101LL/263K samples (a) to (c) described in Table 1. Sample 3 was a further 101LL/263K sample from the same experiment, which showed no PrP-res by immunoblot. The hatched line indicates the level of PrP detected in the 1M GdnHCl insoluble fraction of uninfected 101LL tissue. Capture antibody MAb FH11, detection antibody MAb 7A12. Samples were assayed in duplicate, and the percentage coefficient of variance for each sample pair was $\leq 10$.

**Figure 6. Immunoprecipitation using $PrP^{Sc}$-specific monoclonal antibodies.**
101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to immunoprecipitation (IP) using $PrP^{Sc}$-specific MAbs 89-112 and 136-158 to determine whether forms of $PrP^{Sc}$ which were sensitive to PK were present in these tissues. MAb D13 which precipitates only cellular PrP, and MAb b12 which recognises the HIV gp120 antigen were used as IP controls. Panel A lanes 1-5, 101LL/GSS(h); Panel A lanes 6-10, uninfected 101LL; Panel B lanes 1-5, 101LL/263K(i); Panel B lanes 6-10, uninfected 101LL; Panel C, RML scrapie WT control. Lanes 1&6, crude brain homogenate. Lanes 2&7, IP with MAb D13 (positive control antibody). Lanes 3&8 IP with MAb b12 (negative control antibody). Lanes 4&9, IP with MAb 89-112. Lanes 5&10, IP with MAb 136-158.

**Table 1. Tissues selected for analysis.**
Details of clinical disease and vacuolar pathology in the 5 tissues selected for analysis. All mice showed positive clinical and vacuolar signs of TSE disease and low levels of PrP deposition.

| Tissue used for titration. | Clinical TSE | Vacuolar Pathology | PrP deposition* | Incubation period (days ±SEM) | |
|---|---|---|---|---|---|
| | | | | Primary† | Secondary‡ |
| 101LL/263K(a) | positive | positive | + | 385 | 109 ± 2 |
| 101LL/263K(b) | positive | positive | +/- | 464 | 129 ± 2 |
| 101LL/263K(c) | positive | positive | +/- | 534 | 262 ± 4 |
| 101LL/GSS(d) | positive | positive | + | 259 | 154 ± 3 |
| 101LL/GSS(e) | positive | positive | +/- | 252 | 123 ± 1 |

* scoring of PrP deposition; +++ high, ++ medium, + low, +/- very small grainy deposits.
† Incubation time of each individual mouse on primary transmission of either 263K or P102L GSS.
‡ Incubation time of 101LL mice inoculated with 1% brain homogenate from each specific 101LL/263K or 101LL/GSS infected tissue. Transmission of disease on subpass to 101LL mice was 100% in each case.

**Table 2. Comparison of Titre of infectivity and PrP-res level.**
PrP-res levels, quantified relative to recombinant PrP from digital immunoblot images, and infectivity titre, measured by $ID_{50}$ bioassay. Detection limit of the immunoblot system was estimated to be equivalent to 25μg PrP-res /g wet weight brain.

| Model | PrP-res (μg/g tissue)** | PrP-res (% of ME7) | Titre* (IU/g tissue) |
|---|---|---|---|
| Wt/ME7 | 1994 | 100 | $10^{8.5}$ |
| 101LL/ME7 | 1040 | 52 | $10^{7.8}$ |
| 101LL/263K(a) | 498 | 25 | $10^{8.7}$ |
| 101LL/263K(b) | < 25 | < 1.3 | $10^{7.3}$ |
| 101LL/263K(c) | < 25 | < 1.3 | $10^{7.5}$ |
| 101LL/GSS(d) | < 25 | < 1.3 | $10^{9.8}$ |
| 101LL/GSS(e) | < 25 | < 1.3 | $10^{7.2}$ |

* Titre of infectivity per gram of brain tissue as calculated from $ID_{50}$ bioassay in mice using the Karber calculation.
**The actual amount of PrP-res quantified from the blots (0.5-2mg/g) is higher than would be predicted for mouse tissue, and may reflect the use of recombinant PrP for calibration, as this does not possess any post-translational modifications, and may therefore display altered antibody affinity. However this internal control acts to normalise each blot, and therefore ensures that the relative proportions of PrP-res between each model are real, despite possible errors in the absolute quantification.

Figure 1

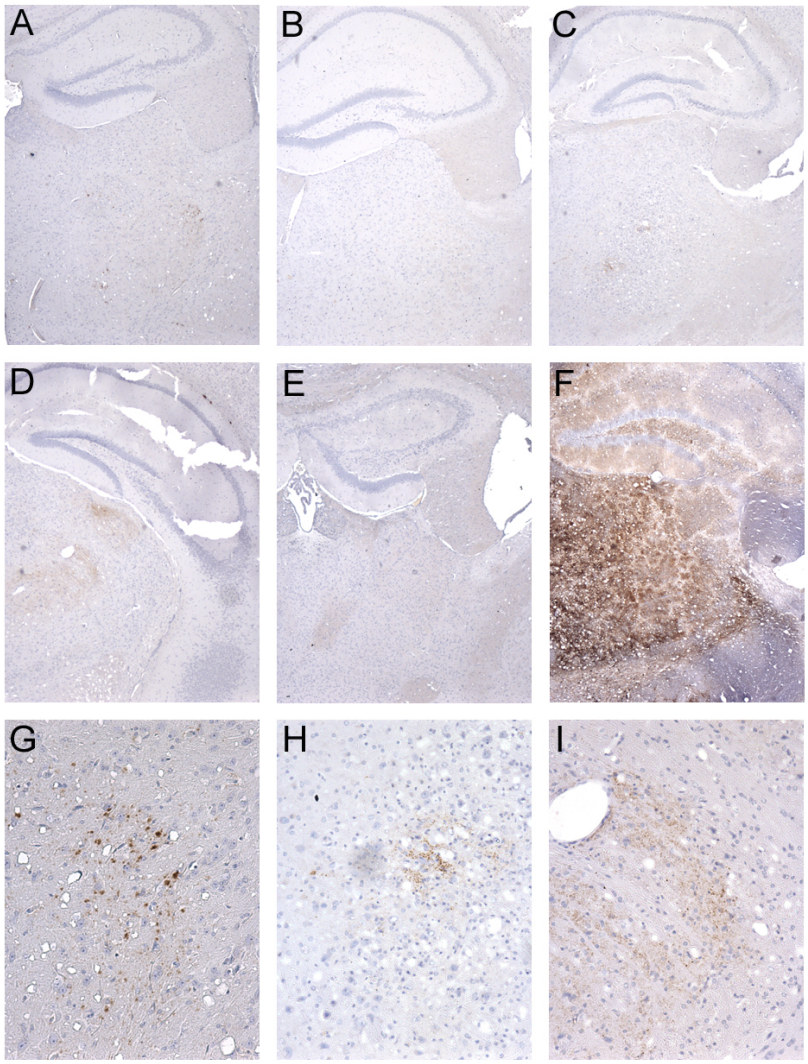

Figure 2

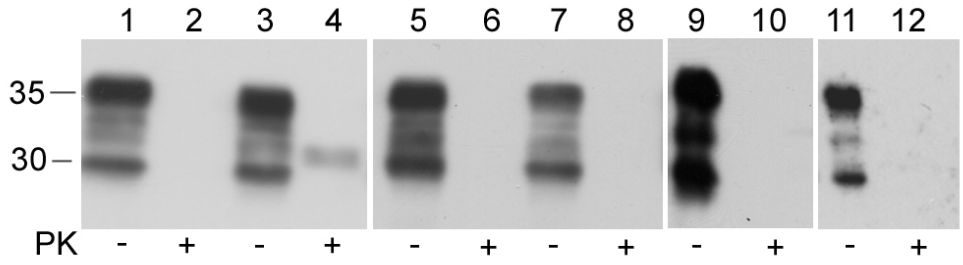

Figure 3

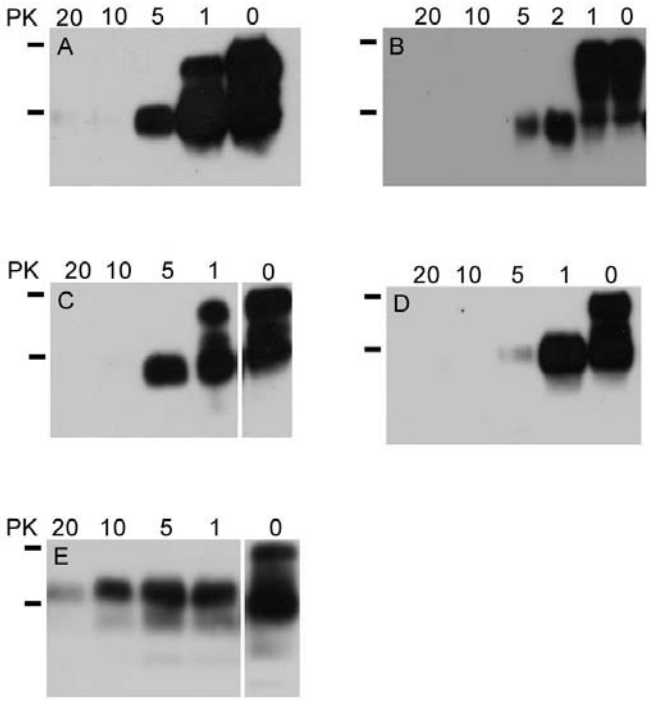

Figure 4

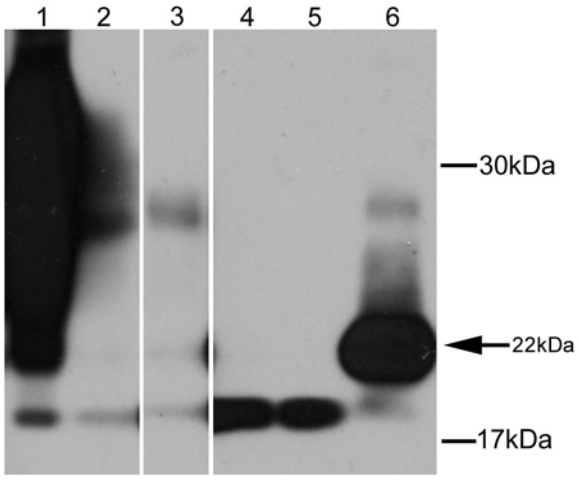

Figure 5

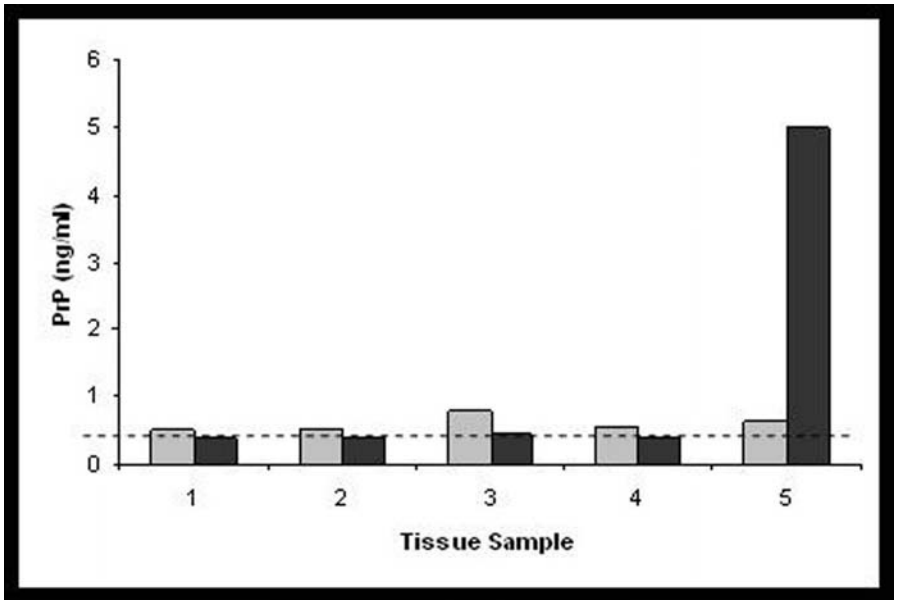

Figure 6