**M03059 Distribution of infectivity and PrPSc in edible tissues of scrapie-infected sheep**
**Technical report**
**(13-12-2010)**

## 1. Background

Scrapie is a transmissible spongiform encephalopathy (TSE) affecting sheep, goats and moufflon. It is transmissible experimentally to ruminants and rodents, but no evidence of transmission to primates has been demonstrated and the assumption is that it is not transmissible to humans. However, atypical scrapie cases have recently been reported in Europe, including in the UK. Current knowledge about this form of the disease is limited and there is insufficient evidence to rule out the possibility of human transmission. Furthermore, there have been recent reports that the BSE agent may have entered the sheep flock in France and Cyprus and the goat flock in France and the UK. These developments emphasise the importance of gaining a precise understanding of the distribution of infectivity in ovine TSEs. There are important differences between animal species in the distribution of the pathological protein isoform PrPSc in TSEs; in field cases of BSE in cattle, PrPSc is restricted to the central nervous system whereas in both scrapie and experimental BSE in sheep PrPSc has been demonstrated in peripheral tissues.

In order to minimise the risk of transmission of TSEs through the food chain, certain tissues are removed from the carcasses of ruminants at slaughter and destroyed. The EU TSE regulation defines the spleen and ileum as specified risk material (SRM) for sheep and goats of all ages whilst the skull, including brain, eyes, tonsils and spinal cord are also considered SRM in animals over 12 months or with permanent incisors. Whilst this approach limits risk based on current knowledge of TSEs, it

could be optimised further by updating information on infectivity in other tissues such as skeletal muscle and viscera.
Sheep naturally infected with scrapie represent an appropriate and useful model to investigate tissue infectivity which may also be extrapolated to other TSE infections. The influence of genetic susceptibility in sheep is well described and the occurrence of natural and experimental scrapie has been linked to polymorphisms at codons 136,154 and 171 of the PrP gene. The effect of such polymorphisms is also dependent on breed and in Suffolk sheep the ARQ/ARQ genotype is considered the most susceptible whilst the ARR/ARR genotype is most resistant to natural disease. The determination of levels of infectivity in tissues from scrapie affected sheep has been hampered by the fact that scrapie isolates have proved very difficult to transmit to mice, due to the species barrier. Recently, the bank vole (Clethrionomys glareolus) has been shown to be highly sensitive to different sources of natural scrapie. After intracerebral inoculation, bank voles succumb to disease following a short incubation time (around 200 days post inoculation) and also show high attack rates. In order to measure infectivity in tissues other than the brain, it is important to determine the limits of detection of the bioassay in titration experiments. Previously to this project we performed two different titration experiments with scrapie isolates from Italy and the UK and showed that bank voles succumb to scrapie after inoculation of 0.02 ml with a 10-5 dilution of brain homogenate.
Since the link between the prion protein and TSEs was first established, several terms have been applied to its pathological form including protease-resistant prion protein ($PrP^{res}$), disease-specific prion protein ($PrP^{d}$) and PrP scrapie ($PrP^{Sc}$). Pathological PrP has the same amino acid sequence as $PrP^{c}$ but differs in its physical properties. Characteristic properties of $PrP^{Sc}$ are protease resistance, insolubility in non-denaturing detergents, hydrophobicity of the polypeptide chain, and retention on the cell surface after cleavage of the GPI anchor. Although $PrP^{Sc}$ is believed

to be the most useful marker of TSE disease identified to date, it is not always directly related to infectivity. For example, the use of certain treatments including sucrose and proteinase K can alter this relationship. Immunohistochemistry (IHC), Western blot and ELISA are the most common monoclonal antibody based techniques for $PrP^{Sc}$ detection used in TSE diagnosis. Several antibodies have been raised against different regions of the prion protein for use in such techniques. We carried out several previous projects which involved Western blotting (WB) and Immunohistochemistry and during these studies a panel of antibodies have been tested using both techniques. The antibodies tested include including 6H4 and 15B3 (Prionics); 2A11 (INIA, Spain); P4 and L42 (R-Biopharm); R145 (VLA Weybridge) and SAF84 (Spi-Bio). Results indicate that the most sensitive of these antibodies for $PrP^{Sc}$ detection were R145 for immunohistochemistry and P4 for western blot of sheep scrapie and L42 for western blot of sheep scrapie and experimental sheep BSE.

The main aim of this project was to define the infectivity and $PrP^{Sc}$ distribution in edible tissues of sheep naturally infected with scrapie and to compare these methods for their ability to give relative quantitation for different tissues.

The Moredun Research Institute (MRI) sheep flock with a high force of natural scrapie infection was the source of tissues and the combined application of both antibody-based techniques and bioassay to edible tissues was used to provide information on their infectivity in TSE infected sheep.

## 2. Selection of cases for transmission

### 2.1 Sheep samples

A total of six Suffolk sheep of the ARQ/ARQ PrP genotype and naturally affected with clinical scrapie at between 22 and 28 months of age were necropsied at MRI and tissues collected as shown in Table 2.1.

| | Abnormal PrP detection | | | | Infectivity |
|---|---|---|---|---|---|
| | Buffered formalin | Tissue macerated and frozen (-80°C) | | | |
| **Ovine tissues*** | **IHC** | **WB** | **ELISA** | **PMCA** | **Inoculum** |
| Brain | 1 BF | 1 WB | 1 ELISA | 1 PMCA | 1 TR |
| Sciatic nerve | 14 BF | 14 WB | 14 ELISA | 14 PMCA | 14 TR |
| Tongue | 32 BF | 32 WB | 32 ELISA | 32 PMCA | 32 TR |
| Prescapular Lymph Node (PsLN) | 45 BF | 45 WB | 45 ELISA | 45 PMCA | 45 TR |
| Distal Jejunal Lymph Node (DJLN) | 51BF | 51 WB | 51 ELISA | 51 PMCA | 51 TR |
| Heart | 62 BF | 62 WB | 62 ELISA | 62 PMCA | 62 TR |
| Liver | 64 BF | 64 WB | 64 ELISA | 64 PMCA | 64 TR |
| Pancreas | 65 BF | 65 WB | 65 ELISA | 65 PMCA | 65 TR |
| Kidney | 66 BF | 66 WB | 66 ELISA | 66 PMCA | 66 TR |
| Semitendinosus muscle | 71 BF | 71 WB | 71 ELISA | 71 PMCA | 71 TR |
| Oculomotor muscle | 76 BF | 76 WB | 76 ELISA | 76 PMCA | 76 TR |
| Cranialis tibialis muscle | 77 BF | 77 WB | 77 ELISA | 77 PMCA | 77 TR |

**Table 2.1 Outline of sample code for ovine tissue collection and assay**
Table shows samples collected from each sheep, the numerical code given to each tissue (e.g. 1 = Brain) and suffix describing the test to be performed on that sample: BF = Buffered Formalin fixative for IHC (immunohistochemistry), WB = Western Blot, ELISA = Enzyme-linked immunosorbent assay, PMCA = Protein Misfolded Cyclic Amplification, Inoculum = Tissues to be transmitted to bank voles. * In addition to the samples listed for test comparison, a wide range of tissues were preserved in buffered formalin for immunohistochemical study.

Immunohistochemistry for abnormal PrP (PrPd) was performed on the collected tissues using the R145 antibody. Of the 6 examined sheep only the first 4 which were found positive were scored for this project. (Animal IDs: 1216B, 1217B, 1221B, 1223B). An identical range of tissues from four control animals were also examined (Animal IDs: N449, N456, N506, N523).

## 2.2 Histology and Immunohistochemistry

### 2.2.1 Methods

Sections stained with haematoxylin and eosin (HE ) were prepared from brain samples taken from all major brain areas including frontal cerebral cortex, corpus striatum, thalamus/hypothalamus, hippocampus, midbrain, cerebellar vermis and medulla oblongata at the obex. Additional blocks of muscle tissue were also stained with HE to determine the presence and distribution of muscle spindles.

Immunohistochemistry (IHC) was performed as follows: paraffin wax embedded tissues were sectioned at 5 μm, mounted on treated glass slides (Superfrost Plus; Menzel-Glaser, City, Germany) and dried overnight at 37ºC. IHC was carried out as described as follow: antigen retrieval included immersion of tissue sections in 98% formic acid for 5 min and autoclaving in 0.2% citrate buffer for 5 min at 121°C. After two blocking steps (to quench endogenous peroxidase activity and to remove non-specific tissue antigens), incubation with the primary antibody was carried out overnight at 4°C. Subsequent steps were performed by a commercial immunoperoxidase technique (Vector-elite ABC kit; Vector Laboratories, Peterborough, UK), after which sections were immersed in 0.5% copper sulphate, to enhance immunoperoxidase colour reaction, and finally counterstained with Mayer's haematoxylin. The R145 antibody was used to detect $PrP^{d}$. R145 recognises the bovine PrP sequence $^{231}$RESQA$^{235}$ and was used at a dilution of 1/2000.

Brain tissue was examined for the presence of vacuolation and the magnitude and distribution of $PrP^d$ was scored. Each of 13 types of $PrP^d$ accumulation are scored in each of 6 different brain regions. The patterns of $PrP^d$ accumulation revealed by $PrP^d$ profiling are consistent for different strains and sources of scrapie.

For ease of recording, the abundance of $PrP^d$ in each visceral tissue was subjectively scored from 0-3 where 0 corresponded to no labelling detected and 1, 2 and 3 corresponded to mild moderate and severe $PrP^d$ accumulation respectively

2.2.2 Results

All 4 scrapie affected sheep showed abundant vacuolation and $PrP^d$ was detected by IHC in brain, in peripheral autonomic system tissues and in lymphoid tissues. See Fig 2.1.

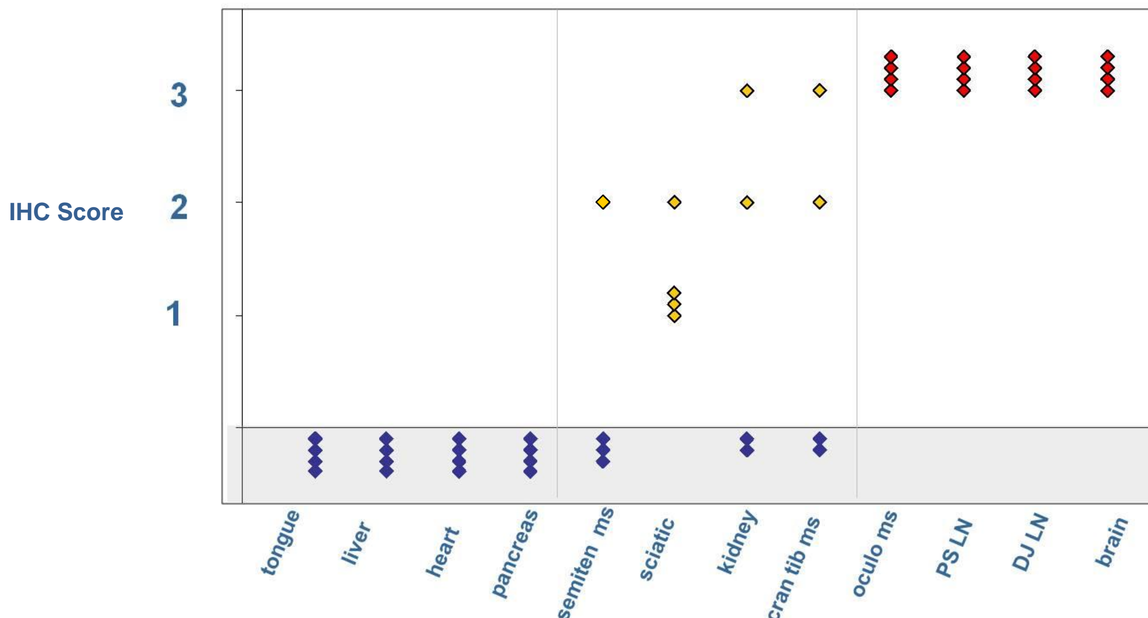



**Fig 2.1 IHC score of $PrP^d$ deposition**
Graph shows the severity of PrPd deposition observed in each tissue sample. Each diamond represents the sample from one animal. Blue indicates tissues with no PrPd; yellow indicates low to moderate levels of PrPd; red indicates high levels of PrPd.

The pattern or profile of $PrP^d$ accumulation in each sheep was typical of that previously observed for Suffolk sheep affected with natural scrapie in the MRI flock and the levels of vacuolation and $PrP^d$ scores were typical of clinical scrapie affected sheep at terminal stages of infection. The levels of vacuolation and the magnitude of the $PrP^d$ profile were similar and did not assist in selection of tissues for transmission studies. Accordingly subjectively determined levels of $PrP^d$ accumulation in viscera, which were more variable than in CNS were used to select animals for further study.

In common with previous studies $PrP^d$ accumulation was found in all the lymphoid tissues examined where it was located almost exclusively in secondary follicles. Except 3rd eyelid, where only one sample was found to contain $PrP^d$, the proportion of $PrP^d$ positive follicles was high with more than 80% of mature secondary follicles showing $PrP^d$ accumulation.  Lymphoid tissues of the gut and pharynx showed the highest proportion of positive secondary follicles. All sheep were positive for $PrP^d$ in the recto-anal lymphoid tissue (RAMALT), a LRS tissue which can be used to provide ante-mortem diagnosis of clinical and pre-clinical disease.

$PrP^d$ accumulation was assessed in peripheral nervous system tissues including, trigeminal, nodose and stellate ganglia and the sympathetic chain. It was also assessed in the Sciatic and vagal nerve trunks and in the mucosal and sub mucosal ganglia of the enteric nervous system. In each of the scrapie affected sheep $PrP^d$ was detected in neurons and satellite cells of each ganglia available for examination and was present at a lower level in vagal and sciatic nerve trunks in 5/6 sheep. $PrP^d$ in nerve trunks was present within myelinated processes. $PrP^d$ was not detected within nerves of the tongue, or in sensory taste receptors. $PrP^d$ was also present in each sheep in the left and right adrenal medulla. Although the cellular location of $PrP^d$ accumulation in the adrenal has not yet been definitively characterised, for the purposes of this

report it is presumed to represent peripheral nerve terminals. Thus each scrapie affected sheep showed widespread $PrP^d$ accumulation within both sympathetic and parasympathetic components of the peripheral nervous system.

$PrP^d$ was also present in spindles of muscles.

In each of the three muscles examined, $PrP^d$ accumulation was confined to muscle spindles and was not detected in myocytes. Muscle spindles sense the degree of stretch of muscle tissue and are abundantly innervated. The frequency of muscle spindles varies markedly between individual muscle tissues. Thus muscle spindles are least frequent in large muscles of the hind limb but are much more frequent in muscles involved in fine motor control such as intra-orbital muscles responsible for movement of the eye. $PrP^d$ was detected in two muscle spindles of the semitendinosis muscle (a large upper hind limb muscles) of one scrapie affected sheep but muscle spindles were not detected in any of the semitendinosis muscles samples taken from the remaining 5 sheep. Muscles spindles were more frequently detected in the cranialis tibialis muscle (a lower hind limb muscle) and $PrP^d$ positive spindles were found in 3 of 6 cranialis tibialis muscles of 3 of 6 scrapie affected sheep. Numerous spindles were detected in the each ocular muscle sample, and in each case $PrP^d$ accumulation was detected in each muscle sample. Thus in Suffolk sheep $PrP^d$ accumulation is a common feature of muscle spindles and the frequency of $PrP^d$ detection in an individual muscle tissue is proportionate to the frequency of spindles in that muscle tissue.

$PrP^d$ within muscle spindles was located in structures resembling small nerves and also within intrafusal muscle cells. Although muscle spindles were consistently associated with $PrP^{d,}$ no evidence of $PrP^d$ accumulation was seen in motor end plates innervating myocytes within the remaining muscle tissue.

Multiple samples of kidney were taken from each sheep and several samples (n>3) specifically included representation of the  renal pelvis. . Only two of the six sheep showed $PrP^{d}$ accumulation in renal pelvis tissues and none showed accumulation in the kidney cortex. This low frequency of $PrP^{d}$ accumulation in kidney pelvis is consistent with previous observations from this flock and from other experimental scrapie infections.

$PrP^{d}$ was not detected in liver, pancreas, myocardium, lung or in the tongue.

No $PrP^{d}$ was detected in any tissue sample taken from either of the two controls.

As described above, the severity of lesions amongst the six sheep examined were highly similar in the brain and did not contribute to selection of tissues for transmission studies. The two sheep with the most widely distributed $PrP^{d}$ in viscera were selected for transmission to bank voles at ISS-Rome.

Furthermore ELISA tissue aliquots from all 4 scrapie cases and one negative control were sent to VLA-Weybridge to be examined under a different project (MO3060).

## 3. Detection of $PrP^{res}$ by Western Blot

Western blot (WB) methods of detection of $PrP^{res}$ are commonly optimised for a study in one tissue type only which has therefore lead to numerous different methodologies being published depending on the species, tissue and TSE agent. The objective of this study was to assess the sensitivity of three western blot methods for detection of $PrP^{res}$ from a range of nervous, lymphatic and muscle tissues from sheep (see Table 1). These included a Sodium phosphotungstic acid (NaPTA) precipitation protocol, a centrifugal concentration protocol and the commercially available BioRad TeSeE WB kit.

3.1 Sample preparation and optimisation of PrP extraction buffer

Previous work at MRI, had developed a protocol for extraction of $PrP^{res}$ from sheep brain material. Brain tissue was homogenised in a brain lysis buffer(BLB; (0.5% NP-40, 0.5% Sodium deoxycholate,Tris buffered saline pH7.4) followed by 37°C/20min incubation with 2% sarkosyl solution, digestion with proteinase K solution (50µg/ml, 37°C/60min) and centrifugal concentration ( 15k rpm/60min/10°C).
This method is adapted from that described by Lee et al (*J. Virol. Methods* 2000:84;77-89) .

In later work with lymphoreticular tissues (lymph nodes) the same protocol did not give satisfactory results and a spleen lysis buffer (SLB; Tris/HCl pH 7.4, 0.1% MgCl, 0.5% N-lauroylsarcosine, 0.1M NaCl, 2%NP-40) was used to homogenise these tissues followed by the same sarkosyl incubation and centrifugal concentration step which gave good results with non-neural tissues such as lymph node and kidney and in our hands was as effective as NaPTA precipitation as first described by Wadsworth et al (*Lancet* 2001;358:171-180). The effect of the lysis buffer used between tissues can be seen in the western blot images below;

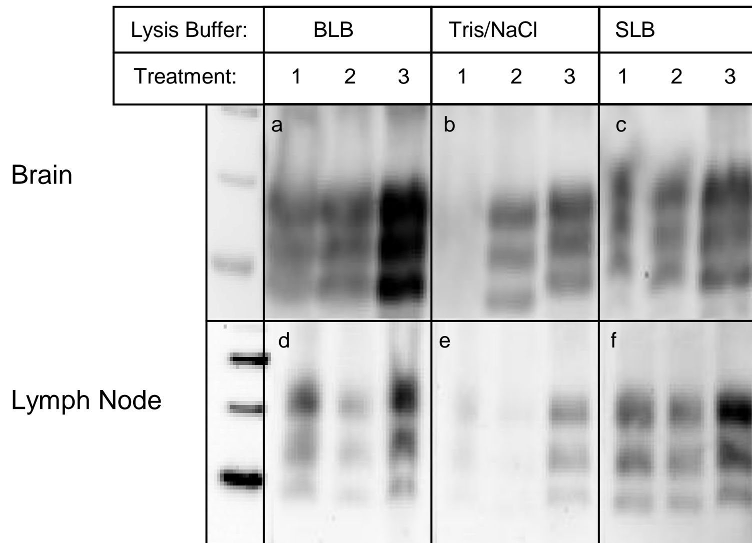



**Fig 3.1.1 Effect of tissue lysis buffer** Figure shows western blot images of PrPres extracted from brain tissue (panels a, b & c) and lymph node tissue (panels d, e & f) using three different lysis buffers (BLB - brain lysis buffer; Tris/NaCL – 0.1M Tris/HCl (pH8.0), 0.1M NaCl); SLB – spleen lysis buffer) Each sample was then used in three different extractions; 1= 15K rpm/1h centrifugation without sarkosyl incubation; 2= 15K rpm/1h centrifugation with 2% sarkosyl incubation 37°C/20min; 3= NaPTA protocol.

BLB was unsuitable for use with lymph node tissue (panel d) as the samples ran poorly on SDS-PAGE giving smeared bands and detection of PrPres was low compared with brain (panel a). A similar result was observed using SLB with brain tissue (panel c) when compared with lymph node (panel f).
Initially in this project BLB was used for the brain and nerve samples and SLB used on the remaining tissues however there were a number of problems encountered at this stage: nerve samples did not run well on SDS-PAGE and required additional centrifugation and washing of pellets in detergent buffer to improve sample preparation, muscle samples formed an unworkable gelatinous precipitate following the sarkosyl incubation step.

At this stage it was decided that in order to make a fair comparison of the intensity of signals between tissue samples on one gel and also between

methods that it was not feasible to use a different extraction method for brain, nerve, muscle and other peripheral tissues before SDS-PAGE. Therefore all FSA project tissues were homogenised in Tris/NaCl buffer (see panels b and d) which gave workable samples from all tissues that ran well on SDS-PAGE and that gave good detection of PrPres, particularly after NaPTA precipitation.

## 3.2. Methods

The WB aliquots of frozen tissue listed in Table 2.1 were used in this part of the study. The tissues from 4 scrapie-infected (Animal IDs: 1216B, 1217B, 1221B, 1223B) and 4 uninfected control sheep (Animal IDs: N449, N456, N506, N523) were tested.

### 3.2.1 Homogenisation

Approximately 200mg of each tissue was weighed into a ribolyser tube and 4 volumes of Tris/NaCl buffer added. Tissues ribolysed at 6.5m/s x 40 sec for two cycles.

### 3.2.2 Centrifugal concentration

300µl of each tissue homogenate were shaken at 37°C with 2% sarkosyl solution then treated with proteinase K solution (50µg/ml) for 1h at 37°C with shaking. Digestion was terminated by adding Pefabloc to 1mM. Samples were then centrifuged at 15,000rpm for 1h at 10°C and pellets resuspended in 50µl 2xSB (Invitrogen) and heated at 100°C/5min prior to SDS-PAGE.

### 3.2.3 NaPTA precipitation

0.5ml of tissue homogenate was made up to 1ml with 4% sarkosyl in Dulbecco's PBS and shaken at 37°C for 10min. Benzonase (50U/ml final concentration) and $MgCl_2$ (1mM final concentration) were added and shaken at 50°C for 30min. Proteinase K added at 50µg/ml and incubated at 50°C for 60min. Add Pefabloc to 1mM to terminate digestion. Pre-warmed (37°C)

NaPTA solution was added to a final concentration of 0.4% and shaken at 37°C for a further 30 min. Samples were then centrifuged at 15,000 rpm for 30min at 10°C and resulting pellets washed in 0.1% sarkosyl/PBS and 250mM Ededtic Acid and re-pelleted at 15,000rpm/15min at 10°C. Final pellets resuspended in 0.1% sarkosyl 50µl 2xSB (Invitrogen) and heated at 100°C/5min prior to SDS-PAGE.

### 3.2.4 SDS-PAGE and western blotting

10µl of sample was loaded on 12% Bis-Tris NuPAGE gels (Invitrogen) run at 150V for 1h. Proteins were electrotransferred onto PVDF membrane at 30V for 1h, blocked in 5% non-fat milk/TBST and probed with antibody P4 (R-biopharm, 1:2000). Signal was detected using Pierce WestFempto chemiluminescent reagents and a Kodak IS440 image station.

### 3.2.5 BioRad TeSeE

All tissue samples were tested using the BioRad TeSeE WB Sheep and Goat kit according to the manufacturer's instructions without modification. The only variable introduced was in the resuspension of some lymph node samples that gave large final pellets.

## 3.3 Results

All control tissues were negative and are not shown in the following graphs or tables.

Each of the following graphs show the signal intensity of chemiluminescent-labelled $PrP^{res}$ measured in western blots of each tissue sample (Fig 3.3.1-2-3). Each diamond represents the sample from one animal. Blue indicates tissues with no detectable $PrP^{res}$; yellow indicates low signal intensity of $PrP^{res}$; red indicates moderate to high signal intensity levels of $PrP^{res}$.

Each western blot image shows an example of the western blot results obtained from a range of tissues from one of the test animals.

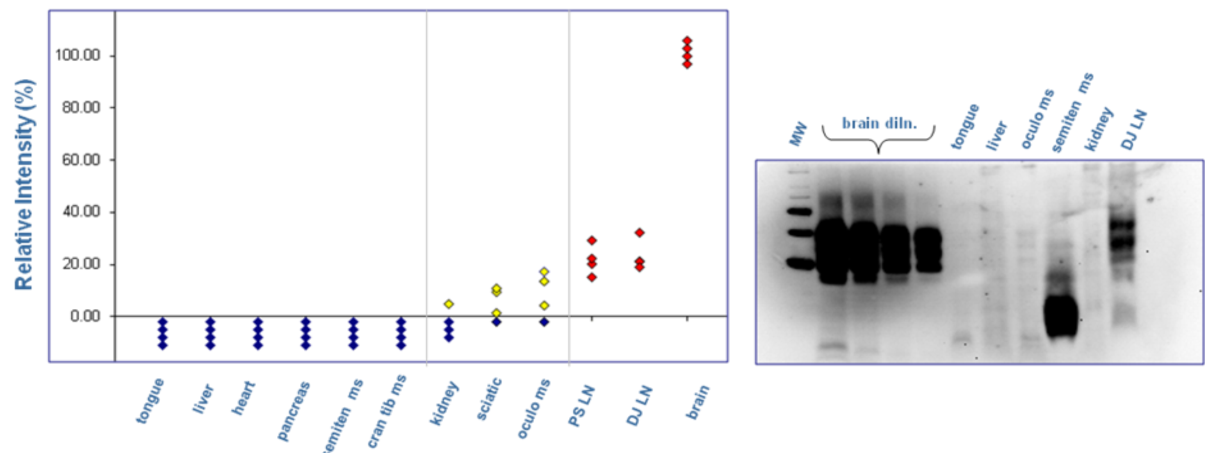



**Fig 3.3.1 Centrifugal concentration**

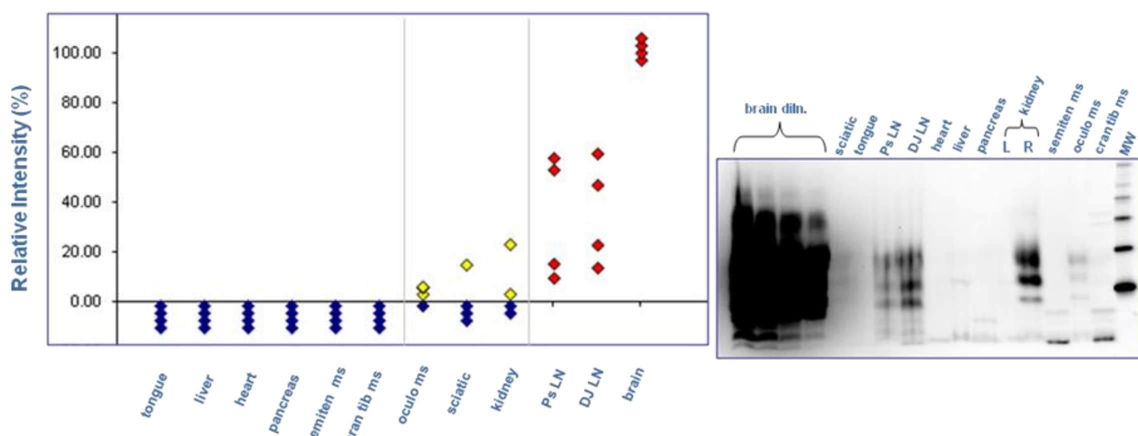



**Fig 3.3.2 BioRad TeSeE WB**

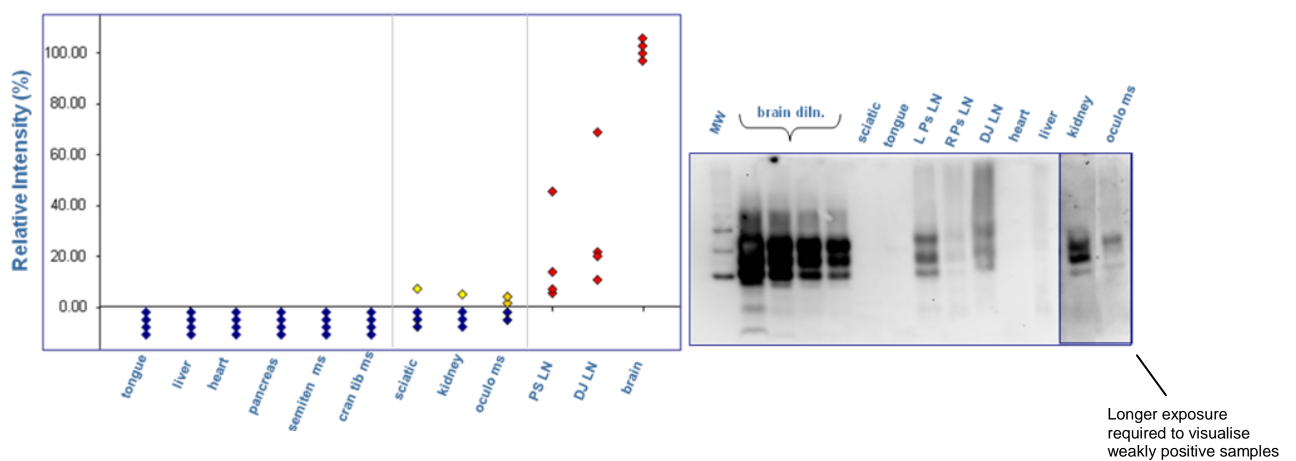



**Fig 3.3.3 NaPTA precipitation**

| | 1216B | | | 1217B | | | 1221B | | | 1223B | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BioRad | Centrifuge | NaPTA | BioRad | Centrifuge | NaPTA | BioRad | Centrifuge | NaPTA | BioRad | Centrifuge | NaPTA |
| Brain | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos |
| Sciatic | neg | pos | neg | neg | pos | pos | neg | neg | neg | pos | pos | neg |
| Tongue | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| PsLN | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos |
| DJLN | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos |
| Heart | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| Liver | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| Pancreas | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| Kidney | neg | neg | neg | neg | neg | neg | pos | neg | neg | pos | pos | pos |
| Semitend ms | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| Oculo ms | pos | pos | pos | pos | pos | pos | neg | neg | neg | pos | pos | neg |
| Cran tib ms | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |

**Tables 3.3.1 & 3.3.2 Western blot detection of PrP$^{res}$**
Table 3.3.1 shows western blot result (pos = positive, neg = negative) for each test tissue in 4 scrapie-infected sheep.Table 3.3.2 summarises table 3.3.1

| | Summary | | |
|---|---|---|---|
| | BioRad | Centrifuge | NaPTA |
| **Brain** | 4/4 | 4/4 | 4/4 |
| **Ps LN** | 4/4 | 4/4 | 4/4 |
| **DJ LN** | 4/4 | 4/4 | 4/4 |
| **Oculo ms** | 3/4 | 3/4 | 2/4 |
| **Sciatic** | 1/4 | 3/4 | 1/4 |
| **Kidney** | 2/4 | 1/4 | 1/4 |
| **Tongue** | neg | neg | neg |
| **Heart** | neg | neg | neg |
| **Liver** | neg | neg | neg |
| **Pancreas** | neg | neg | neg |
| **Semitend ms** | neg | neg | neg |
| **Cran tib ms** | neg | neg | neg |

**Table 3.3.2**

As shown in Tables 3.3.1 and 3.3.2. PrP$^{res}$ was detected in brain, prescapular and distal jejunal lymph nodes by all three western blot methods. Tongue, liver, heart, pancreas and semitendinosus and cranialis tibialis muscles were PrP$^{res}$ negative by all three methods. Sciatic nerve, kidney and oculomotor muscle were the only other tissues to be found positive by one or more of the methods and there was some variability in the detection of PrP$^{res}$ from these tissues for which there are a number of possible explanations.

Sampling: The immunohistochemistry carried out on these samples has shown that PrP$^{d}$ is present in specific locations within tissues such as lymph nodes, kidney, nerve and muscle and a high proportion of a tissue can be negative and therefore gross sampling at post-mortem may not incorporate a PrP$^{d}$ positive area.

Detection limit: The amount of abnormal PrP within these tissues, particularly nerves and muscle appear close to the detection limit of western blot.

Suitability of sample to western blot method: Sciatic nerve was weakly positive in 3/4 animals with the centrifugal concentration method, but 1/4 by BioRad and 1/4 by NaPTA. The one weak positive from the BioRad test was obtained when a larger original tissue weight (400mg) was used, therefore it is possible that had a larger amount of original material been available this may also have been successful using NaPTA as both techniques involve a precipitation step. It is worth noting that nerve material does not have a high mass and a 400mg piece is equivalent to a long piece (approx. 8-10cm) of fresh tissue which is technically challenging to collect at post-mortem.

## 4. Transmission of sheep tissues to bank voles

### 4.1 Assessment of the infectivity in edible tissues by bioassay in bank voles

#### 4.1.1 Methods

All the bioassays in bank voles have been concluded between April and May 2010, by culling all the voles that were still alive at that time (more than two years after inoculation).

The brains of voles, either culled at the terminal stage of disease, for intercurrent disease or at the end of the experiments, were subjected to post-mortem diagnosis by western blot for $PrP^{Sc}$ and by histopathology.

#### 4.1.2 Results

The final results are summarised in Table 4.2.1 (sheep n. 1216B) and Table 4.2.2 (sheep n. 1223B).

Overall, in both sheep analysed, the same 4 edible tissues contained infectivity by vole bioassay: prescapular LN, sciatic nerve, oculomotor muscle and kidney. However, none of these tissues gave 100% attack rate in voles (see tables 4.1.1 and 4.1.2). On the contrary, voles inoculated with heart, tongue or semitendinosus muscle of both sheep did not show clinical signs of disease nor were found positive at post-mortem diagnosis.

| sheep n° 1216B | A<br>Clinically +<br>WB +<br>N (surv. t. range) | B<br>Clinically -<br>WB +<br>N (surv. t. range) | C<br>Clinically -<br>WB -<br>N (surv. t. range) | D<br>Survivors<br>WB-<br>N (time at culling) | attack rate | % | mean (SD) | Prion titre |
|---|---|---|---|---|---|---|---|---|
| Brain $10^{-1}$ | 11 (154-198) | 0 | 0 | 0 | 11/11 | 100% | 176 (13) | 5,01 |
| Brain $10^{-2}$ | 12 (166-305) | 0 | 0 | 0 | 12/12 | 100% | 212 (39) | |
| Brain $10^{-3}$ | 11 (203-358) | 0 | 2 (251, 739) | 2 (868) | 11/15 | 73% | 278 (46) | |
| Brain $10^{-4}$ | 1 (216) | 0 | 10 (302-805) | 2 (868) | 1/13 | 8% | | |
| Brain $10^{-5}$ | 0 | 0 | 9 (432-816) | 5 (868) | 0/14 | 0% | | |
| Brain $10^{-6}$ | 0 | 0 | 7 (202-845) | 3 (868) | 0/10 | 0% | | |
| Sciatic Nerve | 5 (236-697) | 2 (309; 561) | 6 (211-656) | 7 (735) | 7/20 | 35% | 411 (168) | < 3,01 |
| Tongue | 0 | 0 | 5 (211-571) | 5 (831) | 0/10 | 0% | | |
| Prescap. LN | 8 (242-463) | 2 (277; 281) | 3 (298-551) | 1 (805) | 10/14 | 71% | 317 (71) | ~3,01 |
| Heart | 0 | 0 | 9 (391-645) | 4 (805) | 0/13 | 0% | | |
| Kidney | 1 (652) | 1 (670) | 9 (461-767) | 8 (789) | 2/19 | 11% | | ~2,01 |
| Semitend. Ms | 0 | 0 | 10 (369-699) | 8 (789) | 0/18 | 0% | | |
| Oculomotor Ms | 1 (204) | 0 | 10 (228-788) | 6 (830) | 1/17 | 6% | | ~2,01 |

**Table 4.1.1**

| sheep n° 1223B | A<br>Clinically +<br>WB +<br>N (surv. t. range) | B<br>Clinically -<br>WB +<br>N (surv. t. range) | C<br>Clinically -<br>WB -<br>N (surv. t. range) | D<br>Survivors<br>WB-<br>N (time at culling) | attack rate | % | mean (SD) | Prion titre |
|---|---|---|---|---|---|---|---|---|
| Brain $10^{-1}$ | 10 (168-212) | 0 | 0 | 0 | 10/10 | 100% | 187 (13) | 5,35 |
| Brain $10^{-2}$ | 11 (146-477) | 2 (176-186) | 0 | 0 | 13/13 | 100% | 225 (93) | |
| Brain $10^{-3}$ | 10 (225-497) | 1 (456) | 1 (447) | 0 | 11/12 | 92% | 351 (93) | |
| Brain $10^{-4}$ | 2 (274-574) | 1 (362) | 8 (246-837) | 2 (862) | 3/13 | 23% | 403 (154) | |
| Brain $10^{-5}$ | 0 | 0 | 10 (389-798) | 4 (862) | 0/14 | 0% | | |
| Brain $10^{-6}$ | 0 | 0 | 9 (340-789) | 3 (862) | 0/12 | 0% | | |
| Sciatic Nerve | 6 (218-554) | 1 (487) | 6 (280-600) | 2 (835) | 7/15 | 47% | 339 (153) | < 3,35 |
| Tongue | 0 | 0 | 6 (347-789) | 5 (830) | 0/11 | 0% | | |
| Prescap. LN | 11 (189-475) | 2 (250-369) | 4 (202-657) | 0 | 13/17 | 76% | 276 (80) | 3,7 |
| Heart | 0 | 0 | 10 (375-771) | 7 (805) | 0/17 | 0% | | |
| Kidney | 1 (466) | 0 | 10 (204-700) | 6 (785) | 1/17 | 6% | | < 2,35 |
| Semitend. Ms | 0 | 0 | 12 (202-719) | 4 (805) | 0/16 | 0% | | |
| Oculomotor Ms | 6 (328-405) | 0 | 6 (340-747) | 3 (833) | 6/15 | 40% | 376 (28) | < 3,35 |

**Table 4.1.2**

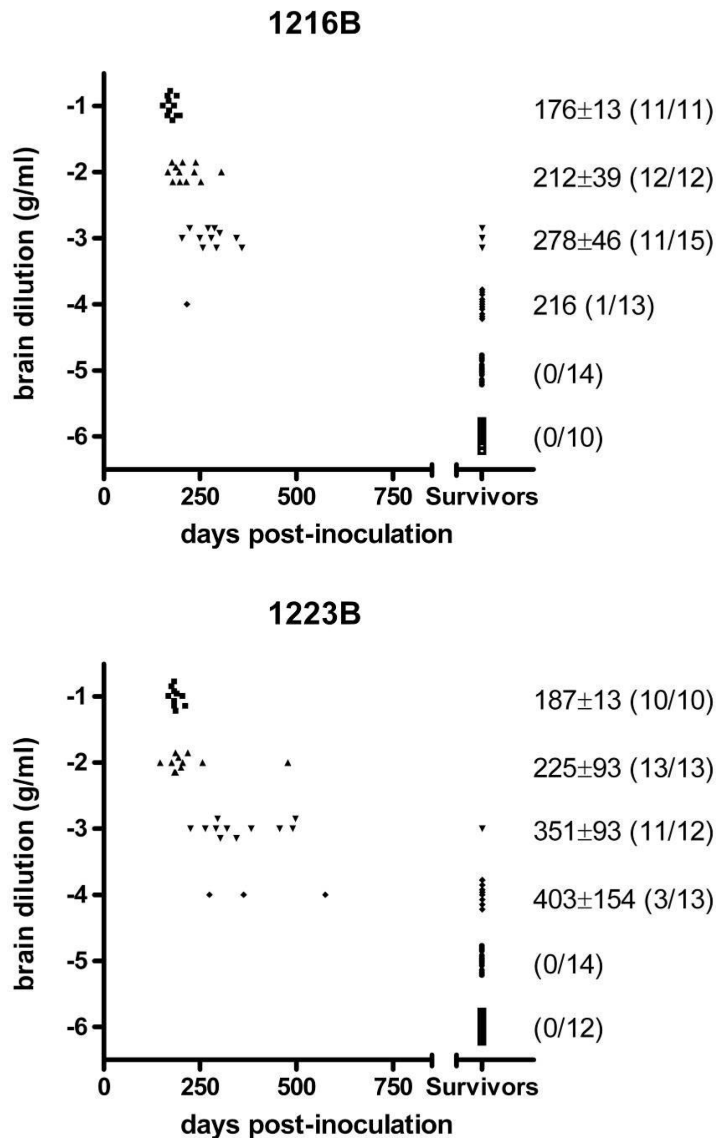
**Table 4.1.1 and 4.1.2 Summary of the vole bioassay results for each sheep tissue set** Column A = clinical cases, Columns B and C = intercurrent deaths
Column D = voles culled at the end of the bioassay, Attack rate = (A+B)/(A+B+C+D)
Prion titre = power of $ID_{50}$ U/g

4.2. Level of infectivity in positive tissues compared to the brain

In order to determine infectivity titre in tissues found to be positive by bioassay we planned to use the incubation time assay. With this method, the infectivity of test tissues is related to a known standard tissue which has been titrated by end-point titration.

The brains of both sheep were used as standard tissues and were titrated by end-point titration, from $10^{-1}$ to $10^{-6}$. The results are summarised in Table 4.1.1 (sheep n. 1216B) and Table 4.1.2 (sheep n. 1223B) and reported in Fig. 4.2.1. Both end-point titrations gave similar results, in that the $10^{-1}$ and $10^{-2}$ dilutions gave 100% attack rate, the $10^{-3}$ and $10^{-4}$ dilutions gave attack rates lower than 100%, while higher dilutions were unable to infect any inoculated vole. The infectivity titres, calculated by the method of Spearmann and Karber, were $10^{5.01}$ i.c. $ID_{50}$ U/g for the brain of sheep 1216B and $10^{5.35}$ i.c. $ID_{50}$ U/g for the brain of sheep 1223B. These results are in line with those previously published for brain tissues from sheep affected by the same scrapie strain used in this project (Di Bari et al, 2008).

Non-linear regression analysis of prion titre against mean survival time (± sd) of voles inoculated with serial dilutions of 1216B and 1223B were performed (Fig. 4.2.2). Similar relations between prion titre and survival time were observed for the two titrations.




.

**Fig. 4.2.1. End point titration of scrapie infectivity in voles.**

Serial dilutions of brain homogenates from 1216B (top panel) and 1223B (bottom panel) scrapie isolates were inoculated intracerebrally in voles. Symbols represent individual survival times. The diseased voles were positive for brain $PrP^{res}$. Inoculated voles that were not infected after 868 d.p.i. (top panel) or 862 d.p.i (bottom panel) are plotted in compressed form after the *x* axes break point. Voles culled with intercurrent disease at >200 d.p.i. and negative by WB are plotted as survivors. The mean survival time (days ± sd) and the number of diseased/inoculated voles are indicated on the right of each chart.

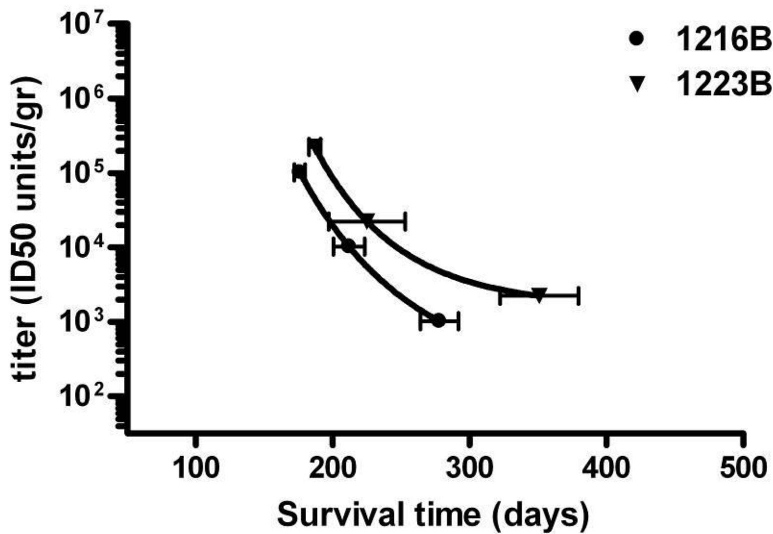



**Fig 4.2.2. Non-linear regression analysis** prion titre against mean survival time (± sd) of voles inoculated with serial dilutions of 1216B (closed circles) and 1223B (closed triangles).

Given that none of the positive edible tissues gave a 100% attack rate, their level of infectivity could not be estimated by the incubation time assay. We decided to estimate the titre of prescapular LNs which gave at least 70% attack rate in both sheep. Unfortunately, the survival time of voles infected with the prescapular LN of sheep 1216B was outside the range of those observed for the brain dilutions of the respective brain, and thus was not estimated. On the contrary, the survival time for the prescapular LN of sheep 1223B was within the range of the brain dilutions of the respective brain and the estimated titre was $10^{3.7}$ i.c. $ID_{50}$ U/g, around two order of magnitude less than that measured in the brain.

For all other positive edible tissues the infectious titre was estimated by visual inspection of the survival curves in comparison with those obtained from the brain dilutions of the respective brains (Fig. 4.2.3) and are reported in tables 4.1.1 and 4.1. 2. For sheep 1216B, the prescapular LN, sciatic nerve, kidney and oculomotor muscle gave survival curves which were between $10^{-3}$ and $10^{-}$

$^{4}$ brain dilutions and their titres were thus comprised between $10^{3.01}$ and $10^{2.01}$ i.c. $ID_{50}$ U/g, i.e. 2-to-3 orders of magnitude less than the brain. However differences among them were quite evident, with the prescapular LN showing a survival curve very similar to the $10^{-3}$ brain dilution, the kidney and the oculomotor ms showing survival curves very similar to the $10^{-4}$ brain dilution and the sciatic nerve being intermediate (Fig. 4.2.3).
For sheep 1223B, the sciatic nerve and oculomotor muscle gave survival curves which were between $10^{-3}$ and $10^{-4}$ brain dilutions and their titres were thus comprised between was $10^{3.35}$ and $10^{2.35}$ i.c. $ID_{50}$ U/g, i.e. 2-to-3 orders of magnitude less than the brain, while the kidney contained less than $10^{2.35}$ i.c. $ID_{50}$ U/g.

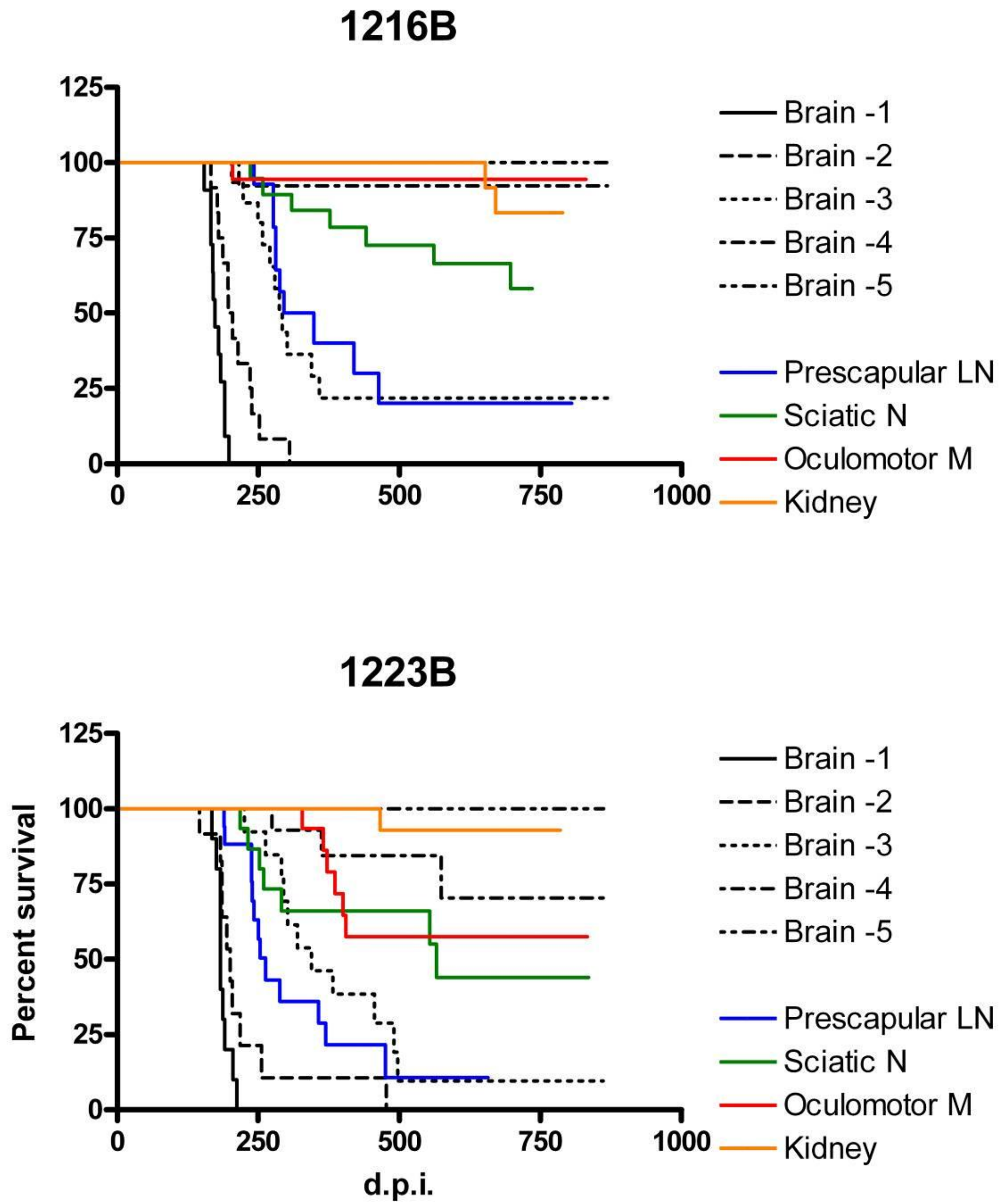



**Fig 4.2.3. Survival curves obtained from positive edible tissues of sheep** 1216B (top panel) and 1223B (bottom panel) in comparison with the respective brain dilutions.

### 4.3. Level of $PrP^{Sc}$ in the inocula by Western Blot

During the project it became evident that there may be differences between samples from the same organ and to make the interpretation of the result as consistent as possible it was agreed to use the inocula as a substrate for the WB to be compared to the bioassay. This part of the project was carried out using funding from Istituto Superiore di Sanita' (Rome) using a method very similar to the centrifugation methods used at MRI.

#### 4.3.1 Methods

$PrP^{Sc}$ contained in the inocula used to infect voles was detected by WB using a previously published method, which showed high sensitivity for $PrP^{Sc}$ detection in peripheral tissues (Vascellari et al, 2007). For WB analysis of the inocula, 1 ml of tissue homogenates (10% w/v in PBS pH 7.4) were added with 110 µl of PBS containing 20% Sarcosyl (Sigma) to obtain a final concentration of 2% and incubated for 20 min at 37°C with gentle shaking before proteinase K (50 µg/ml) digestion for 60 min at 37°C with gentle shaking. Protease treatment was stopped with PMSF (Sigma) (33 µl of a stock solution, to give a final concentration of 3 mM) and the treated homogenates were centrifuged at 20,000 g for 60 min at 20°C. Pellets were dissolved in 40 µl of NU-PAGE sample buffer (Invitrogen), heated at 95 °C for 10 min and centrifuged in a microcentrifuge at 12,000 rpm for 5 min. Twenty µl of the supernatants (50 mg tissue equivalent per lane) were loaded onto 12% bis-Tris polyacrylamide gels (Invitrogen). Electrophoresis was carried out at 200 V

for 40 min and WB performed on PVDF membranes (Millipore). The blots were blocked in PBS containing 0.1% Tween 20 and 3% non-fat milk powder for 1 h. $PrP^{Sc}$ was detected with the monoclonal antibody P4 (0.4 µg/ml, R-Biopharm) for 60 min at room temperature. Peroxidase-conjugated goat anti-mouse IgG (1:80,000 for 1 h, Pierce) was used as secondary antibody. The membranes were developed with the SuperSignal West Femto enhanced chemiluminescence method (Pierce). Chemiluminescence was detected with the VersaDoc imaging system (Bio-Rad).

The experiment was repeated 3 times and in all experimental sessions the respective brain dilutions were analysed in comparison with the edible tissues. The results of these experiments are summarised in table 4.3.1.

| 1216B | WB inocula | Bioassay | 1223B | WB inocula | Bioassay |
|---|---|---|---|---|---|
| Brain -1 | pos | pos | Brain -1 | pos | pos |
| Brain -2 | pos | pos | Brain -2 | pos | pos |
| Brain -3 | pos | pos | Brain -3 | pos | pos |
| Brain -4 | neg | pos | Brain -4 | neg | pos |
| Brain -5 | neg | neg | Brain -5 | neg | neg |
| Brain -6 | neg | neg | Brain -6 | neg | neg |
| Sciatic Nerve | ++ | ++ | Sciatic Nerve | ++ | ++ |
| Tongue | neg | neg | Tongue | neg | neg |
| Prescap. LN | +++ | +++ | Prescap. LN | +++ | +++ |
| Heart | neg | neg | Heart | neg | neg |
| Kidney | neg | + | Kidney | + | + |
| Semitend. Ms | neg | neg | Semitend. Ms | neg | neg |
| Oculomotor Ms | + | + | Oculomotor Ms | ++ | ++ |

**Table 4.3.1 Comparison of WB and bioassay results**

For the brain dilutions, only positive and negative dilutions are indicated. For the edible tissues a semi-quantification is provided.

The WB signal was semi-quantified as follows:

+++ = a tissue positive in all experiments and with a WB signal between brain dilutions -2 and -3

++ = a tissue positive in at least 2/3 experiments and with a WB signal below brain dilution -3

+ = a tissue positive in 1/3 experiments and with a WB signal below brain dilution -3

The bioassay results were categorised as follows:

+++ = between brain dilutions -2 and -3 or similar to -3

++ = between brain dilutions -3 and -4

+ = similar to brain dilution -4 or less

In both sheep, the last positive brain dilution was $10^{-3}$, which suggest that the WB was less sensitive than the vole bioassay (the last positive dilution by bioassay was $10^{-4}$). Despite this lower sensitivity, $PrP^{Sc}$ was detected in all tissues positive by vole bioassay with the exception of the kidney from sheep 1216B which was negative by WB. However, the only tissue which was found positive in all experiments was the prescapular LN, which showed a $PrP^{Sc}$ amount intermediate between brain dilutions $10^{-2}$ and $10^{-3}$. In all other positive tissues $PrP^{Sc}$ levels were near the limit of detection of our WB, being positive in only 1/3 or 2/3 experiments and with very low signals.

Semi-quantification of the WB signal was attempted by comparing the level of $PrP^{Sc}$ in the tissues with those observed in the brain dilutions (see tables 4.3.1 and 4.3.2). This analysis showed that the levels of infectivity in tissues by bioassay correlate well with the semi-quantification of $PrP^{Sc}$.

In both sheep analysed prescapular LN, sciatic nerve, oculomotor muscle and kidney showed detectable levels of scrapie infectivity by vole bioassay. The levels of infectivity were rather low, being 2-to-3 orders of magnitude less than in the brain. The prescapular LN contained the highest levels of infectivity, followed by the sciatic nerve, the oculomotor muscle and the kidney.
The relative levels of infectivity of edible tissues compared to the brain were in good agreement with the relative levels of $PrP^{Sc}$ determined by WB in the same inocula.

## 5. Conclusions

1. There was a good correlation between laboratory techniques and bioassay results.
2. Discrepancies were observed in tissues with very low levels of abnormal PrP.
3. Sampling is critical to avoid potential false negative rapid tests and bioassay results

*References*


- Di Bari, M.A., Chianini, F., Vaccari, G., Esposito, E., Conte, M., Eaton, S.L., Hamilton, S., Finlayson, J., Steele, P.J., Dagleish, M.P., Reid, H.W., Bruce, M., Jeffrey, M., Agrimi, U., Nonno R., 2008. The bank vole (Myodes glareolus) as a sensitive bioassay for sheep scrapie. Journal of General Virology **89**, 2975–2985.
- Vascellari, M., Nonno, R., Mutinelli, F., Bigolaro, M., Di Bari, M.A., Melchiotti, E., Marcon, S., D'Agostino, C., Vaccari, G., Conte, M., De Grossi, L., Rosone, F., Giordani, F., Agrimi, U., 2007. PrPSc in Salivary Glands of Scrapie-Affected Sheep. Journal of Virology **81,** 4872-4876.