

# **FINAL REPORT**

**The tissue distribution of disease-related PrP and infectivity for atypical scrapie in sheep following experimental oral challenges**

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## Introduction

Atypical scrapie first appeared as cases of Nor 98 in Norway and other parts of the EU (Benestad et al 2003) in sheep with genotypes thought to be relatively resistant to classical scrapie, for example those encoding the AHQ and ARR PrP gene alleles. Further reports found that a form of disease-related PrP protein could be recovered from the brains of sheep of ARR/ARR genotypes in Germany following abattoir testing (Buschmann et al 2004). On Western blots the atypical PrP protein shows a characteristic low molecular weight band(s) of 10-12kD and a high sensitivity to proteinase K treatment (Le Dur et al 2005). In France brain recovered from atypical cases in sheep of ARR/ARR genotype were transmitted to Tg338 mice with incubation periods of around 200 days, thus demonstrating the transmissible nature of this class of TSE (Le Dur et al 2005). Tg338 mice are highly sensitive to atypical scrapie and show similar pathology and PrP characteristics to those seen in sheep (Andreoletti et al 2011). Atypical scrapie has also been transmitted experimentally by the intracerebral route to sheep although this has proven not to be easy with less than 100% of clinical cases even in fully susceptible sheep (Simmons et al, 2007 and 2010). Meanwhile the National Scrapie Plan (NSP) in the UK aimed to decrease frequencies of genotypes susceptible to classical scrapie and to BSE, including the VRQ allele, and has inevitably had the effect of increasing frequencies of sheep with genotypes which are now being found in association with atypical scrapie. As a result there is considerable concern about the potential risks of this apparently novel infection.

In 2007 when this project was started, very little was known about distribution of infectivity within the body tissues of atypical scrapie. The existing Specified Risk Materials (SRM) regulations within Great Britain result in the removal from the human food chain of spleen and ileum from all ages of sheep and skull, including brain and eyes, tonsils and spinal cord from animals over 12 months of age (FSA web site at <http://www.food.gov.uk/policy-advice/bse/controls>). However because of the shortage of sheep available for study of atypical scrapie pathogenesis, it was not known at the start of this project whether the SRM regulations will protect consumers from any risk associated with atypical scrapie. There is a shortage of sheep brain from atypical scrapie animals as most cases have been found during

rapid surveillance testing which is not conducive to collection and storage of large amounts of tissue suitable for transmissions. However this project took advantage of the fact that the NPU Cheviot flock (now held at The Roslin Institute) has had several cases of atypical scrapie within it (Foster et al 2008) allowing us easily to carry out transmissions with fully characterised material. In addition as atypical scrapie has been difficult to transmit experimentally to sheep, we infected the sheep at very young ages, before weaning. From our previous TSE pathogenesis studies, it is clear that this age group is particularly susceptible to infection (Hunter et al 2012). Atypical scrapie transmissions to young lambs were therefore carried out, followed by bioassay of inoculated sheep brain, spleen, liver and muscle in tg 338 mice. This proposal was aimed at providing information relevant to the requirements of the Food Standards Agency which wishes to assess how effective the SRM controls are in reducing any potential human risks from atypical scrapie.

## Methods

### General statement

Many of the methods described below are standard in our laboratory and have been developed over decades of study. For some methods, further development and optimisation was required and this is indicated as appropriate.

### Ethical approval

All of the experiments using animals in this project were subject to consideration and approval by the local Ethical Review Committee at The Roslin Institute. All studies are covered by Home Office Project and Personal licences.

### Sheep

Sheep of AHQ/AHQ and AHQ/ARQ genotype were chosen from within the NPU Cheviot flock. This flock has been described in detail previously (Hunter et al 1996) but in short, was formed in 1960 and closed to new introductions shortly thereafter. It has a long history in scrapie research, particularly in connection with the elucidation of PrP genetics and links with scrapie and BSE susceptibility. For this experiment, animals had to be bred specifically as very young lambs were required.

### Sheep genotyping method

Various mating groups were set up in order to maximise the chances of lambs being born of the required genotypes AHQ/AHQ and AHQ/ARQ. Since within the NPU Cheviot flock there is a limited group of animals with the necessary genotypes for parents, this took 2-3 years, but this was incorporated into the project planning.

Blood was collected from likely lambs as soon as possible after birth and PrP genotyping was performed on PCR-amplified DNA fragments generated from genomic DNA which was extracted from the blood samples. In short the genotyping method was as follows: buffy coat samples were digested with 7mg/ml proteinase K (Qiagen, UK) in 600µl buffer (0.34% SDS, 0.34mM EDTA, 3.4mM TRIS pH8.0, 0.1M NaAcetate, 0.33xPBS) at 37°C for 1-5 hours or overnight. Protein was then removed from DNA by standard phenol/chloroform extraction. Polymerase chain reaction (PCR) amplification of isolated DNA (50-100ng) was performed with Sigma JumpStart™ REDTaq® DNA polymerase and buffers, 200 µM (each) dNTPs (Roche, Switzerland) and 0.4 µM of each oligonucleotide primer: PS-141d GGAATGTGAAGAACATTTATGACCTAGAAT and PS+109u CAAGAGAGAAGCAAGAAATGAGACA. PCR conditions were as follows: 3min at 95°C followed by 40 cycles of 30sec at 95°C, 30sec at 61°C and 1min at 72°C. A final elongation step of 10min at 72 °C concluded PCR. 1-3 µl purified PCR fragment were sequenced using oligonucleotide PS+50u CCCCCAACCTGGCAAAGATTAAGA and BigDye® terminator v3.1 cycle sequencing kit. Purified reactions were run on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, USA) and the DNA sequence read automatically but confirmed by eye.

### Atypical scrapie

The source of atypical scrapie brain was sheep number 51x45 which was the index case within the NPU Cheviot flock although since that finding (Foster et al 2008) we have found several other cases from the years before and after that animal was discovered. It was of genotype AHQ/AHQ and was culled at 97 months of age in 2001 as it had clinical signs suggesting a nervous disorder with intermittent shaking/trembling of the head, shoulders and flanks which lasted for around 4 months, very much longer than classical scrapie clinical signs in our experience.

PrP protein preparations from the brain of 51x45 show the characteristic 10-12kD band on Western blot and the brain pathology showed by immunohistochemistry using BG4 (anti PrP monoclonal antibody) a pronounced staining in the cerebellum, also characteristic of atypical scrapie. We have also transmitted 51x45 to tg 338 mice and obtained the same lesion profile and protein characteristics described by others (examples shown in Appendix 1).

#### Sheep dosing method

Lambs with specific PrP genotypes were dosed by intra-buccal (oral) challenge with 5mls of  $10^{-1}$  homogenate of brain from atypical scrapie case 51x45. The lambs were between 5 and 7 weeks old and penned as a single group after being weaned from their dams following challenge. The lambs were returned to their mothers in one pen after challenge and weaned at the appropriate time of 2-3 months of age.

Control lambs were selected and weaned when ready from their dams. Dosed and control lambs were penned separately until end point of the experiment.

#### Observation for clinical signs in the sheep

Sheep were penned indoors and were fed and observed each day. An assessment of general health was made by the animal carer and any problems noted and reported to the named veterinary surgeon. TSE-like signs result in culling of the sheep according to Home Office regulations (Schedule 1 procedure). Intercurrent illness is managed according to the advice of the veterinary surgeon and if the animal does not respond to treatment is culled as above. All animals are assessed for TSE pathology whatever the cause of death unless the sheep dies during the night and is found dead. In the latter case brain autolysis could prevent satisfactory analysis.

#### Post mortem tissues collection

The following tissues were collected from each sheep which was culled. Sterile (suitable for transmission to mice or sheep) and frozen: brain, spleen, kidney, liver, jejunum, semitendinous muscle (m), semimembranosus m.; longissimus m.

Flash frozen and suitable for biochemical analysis: brain, spleen, kidney, liver, jejunum, semitendinous muscle (m), semimembranosus m.; longissimus m., distal ileum, prescapular lymph node (ln), prefemoral ln, mesenteric ln.

Fixed tissues (suitable for histology): brain, spleen, kidney, liver, jejunum, semitendinous muscle (m), semimembranosus m.; longissimus m., distal ileum, prescapular lymph node (ln), prefemoral ln, mesenteric ln.

In the rare instances when a sheep dies suddenly with no warning, especially at night and if found dead, an emergency tissue list is standard for all our experiments as many, if not most, of the tissues will be in a less than perfect condition. The emergency list is for frozen and fixed: brain, spleen, tonsil and one lymph node. This actually occurred with one single animal in this project and its details are with the results.

#### Preparation of inoculum

Strict procedures are adhered to at The Roslin Institute to ensure that tissues, homogenates, lysates and cell preparations used for inoculation/injection are free from microbial contamination.

Following removal, tissues or cell preparations are either stored or aseptically prepared for inoculation. All manipulations take place within a dedicated class II safety cabinet. When possible the laboratory within which the tissues are removed is not used simultaneously for other purposes at the same time to avoid opportunities for accidental microbiological contamination.

Experience has shown that if the above protocols are closely adhered to microbial contamination-free inocula preparations can be routinely achieved. However it is routine also to check sheep samples for bacterial contamination by testing growth on agar plates prior to inoculation. Contaminated samples are not be used for mouse inoculation.

#### Mouse inoculation

In order to detect infectivity in our sheep experimentally infected with atypical scrapie, transmissions to tg 338 mice, which express the sheep VRQ PrP gene allele, were carried out from four tissues: brain, spleen, liver and longissimus dorsi (muscle from the back of the animal). Tg 338 mice are very sensitive to atypical scrapie which is unexpected due to their genotype. Andreoletti et al (2011) carried out a titration of atypical scrapie in these mice and found  $>10^9$  ID<sub>50</sub> units/g in brain tissue making the mice ideal for bioassaying what might be low titres of infectivity. Homogenates (10%) were made in saline from each of the chosen sheep tissues and firstly were checked on agar plates for bacterial contamination (two horse blood agar plates, one aerobic, one anaerobic, 37C for 3-4 days). Contaminated samples are not injected into animals. In this project there was no contamination of the atypical scrapie challenged sheep tissues. Homogenates were stored frozen at -20C until proven sterile.

Tissue homogenates, if proven sterile, are injected by two routes per mouse: intracerebrally (0.02ml) and intraperitoneally (0.1ml) which is the standard route we use to maximise the likelihood of infection from primary transmissions and allows comparison between different experiments.

#### TSE symptoms and scoring in mice

Mice are cared for using standard procedures. In summary, animals are checked daily, and scored weekly by trained observers for TSE signs. There is a formalised scoring system, covered by Home Office licences, for classification of TSE clinical signs. Mice suspected of having TSE clinical signs are killed by cervical fracture. Diagnosis is confirmed by pathology.

#### Pathology methods

Sample coding: Each animal is given an anonymous number, referred to in house as a BP number. This ensures the treatment and more importantly the examination and scoring of tissues is done in a blinded fashion. Mouse tissues have a number



prefixed with the letter B and sheep with the letter S. The pathology scoring is described later but it is carried out as the animals come into the system which means that the recording system has a mix of all concurrent experiments. The PI decodes the experiment and pulls out the particular samples required for one experiment into another data file, usually Excel.

### Fixation

Fixation of fresh tissues requires the following chemicals: 10% formal saline, PLP (Paraformaldehyde, lysine, periodate) and fixative (Periodate-lysine-paraformaldehyde).

Mouse tissues were fixed for a minimum of 48 hours in 10% formal saline, enough to cover the tissues, decanted and then replace with 10% formal saline. Ovine tissues were fixed in 10% formal saline for a minimum of 7 days prior to being trimmed. Tissues were washed in running water for 1-2 hours before further processing.

### Trimming of fixed tissues.

Mouse brains were removed from fixative and trimmed into the five specific pieces to allow standard vacuolation scores to be given by expert staff to specific areas of the brain from which a lesion profile is generated (see Appendix 2). Ovine brains were trimmed into blocks A-H as in Appendix 3. Intestinal and other epithelial tissues were trimmed and embedded on edge to show the layers in the tissues.

### Tissue processing to wax blocks

Tissues are processed in automated tissue processors (eg Leica TP1050) according to tissue type and size using the appropriate schedule. The reagents required are 10% formal saline, fixatives e.g. 10% formal saline, 70, 90, 95 and 99% IMS

(industrial methylated spirits), Xylene and Paraffin wax – e.g. Tissue-Tek 111. The principle is that fixed tissues are processed through ascending grades of IMS and Xylene to paraffin wax to render the tissues suitable for sectioning. Tissues are fixed for a minimum of 24 hours.

Operation of Shandon Excelsior (a) Mouse processing schedules

Station	Shandon Excelsior	TP1050	Routine (TP1050)	Routine (Shandon)	PLP (day run) (TP1050)	PLP (day run) (Shandon)	PLP (Short Day run) Shandon	Temp.
			7 hr 20m	7 hr 20m	5 hr 37m	5 hr 12m	4 hr 47m	
1	10% formal saline	10% Formal saline	5 mins	5mins	0	0	0	ambient
2	70% IMS	70% IMS	40 mins	40 mins	30 mins	30 mins	5 mins	ambient
3	90% IMS	90% IMS	40 mins	40 mins	30 mins	30 mins	30 mins	ambient
4	95% IMS	95% IMS	40 mins	40 mins	30 mins	30 mins	30 mins	ambient
5	99% IMS	99% IMS	40 mins	40 mins	35 mins	35 mins	35 mins	ambient
6	99% IMS	99% IMS	40 mins	40 mins	35 mins	35 mins	35 mins	ambient
7	99% IMS	99% IMS	30 mins	30 mins	30 mins	30 mins	30 mins	ambient
8	Xylene	Xylene	30 mins	30 mins	30 mins	30 mins	30 mins	ambient
9	Xylene	Xylene	30 mins	30 mins	20 mins	20 mins	20 mins	ambient

								t
10	Xylene	Xylene	30 mins	30 mins	20 mins	20 mins	20 mins	ambien t
11	Wax	Paraffin wax	25 mins	25 mins	10 mins	20 mins	20 mins	61
12	Wax	Paraffin wax	25 mins	25 mins	15 mins	10 mins	10 mins	61
13	Wax	Paraffin wax	25 mins	25 mins	15 mins	10 mins	10 mins	61
Clean cycle			1 hour	Extended flush	1 hour	Extended flush	Extende d flush	
Clean 1	Xylene	Xylene	9 cycles	30 mins	9 cycles	30 mins	30 mins	
Clean 2	99% IMS	99% IMS	6 cycles	5 mins	6 cycles	5 mins	5 mins	
Clean 3	Water	water	2 cycles	2 mins	2 cycles	2 mins	2 mins	

(b) Sheep processing schedules

Statio n	Shandon Excelsio r	TP1050	Sheep (TP1050 )	Sheep (Shando n excelsio r)	Hamster Formal saline (TP1050)	Hamster Formal saline (Shandon excelsior)	Temp.	
			18 hr 45m		13hr 30m	13hr 30m		
1	10% formal saline	10% Formal saline	5 mins	5 mins	5mins	5mins	ambient	
2	70% IMS	70% IMS	2hours	2hours	1.5 hours	1.5 hours	ambient	
3	90% IMS	90% IMS	2hours	2hours	1.5 hours	1.5 hours	ambient	
4	95% IMS	95% IMS	2hours	2hours	1.5 hours	1.5 hours	ambient	
5	99% IMS	99% IMS	2hours	2hours	1.5 hours	1.5 hours	ambient	

6	99% IMS	99% IMS	2hours	2hours	1.5 hours	1.5 hours	ambient	
7	99% IMS	99% IMS	1h45mins	1h45mins	45mins	45mins	ambient	
8	Xylene	xylene	1h45mins	1h45mins	45mins	45mins	ambient	
9	Xylene	Xylene	1hour	1hour	45mins	45mins	ambient	
10	Xylene	Xylene	1hour	1hour	45mins	45mins	ambient	
11	Wax	Paraffin wax	50mins	50mins	45mins	45mins	60	
12	Wax	Paraffin wax	50mins	50mins	45mins	45mins	60	
13	Wax	Paraffin wax	50mins	50mins	45mins	45mins	60	
Clean cycle			1 hour	1 hour	1 hour	1 hour		
Clean 1	Xylene	Xylene	9 cycles	9 cycles	9 cycles	9 cycles		
Clean 2	99% IMS	99% IMS	6 cycles	6 cycles	6 cycles	6 cycles		
Clean 3	Water	water	2 cycles	2 cycles	2 cycles	2 cycles		

### Section cutting

Firstly the face of the paraffin wax blocks is trimmed to ensure that the tissue surface is adequately exposed and the cutting surface of the block softened using ice and water prior to section cutting. Ribbons of sections, normally 5 or 6 microns thick are cut and transferred to a waterbath. Tissue from one animal only is floated on the waterbath at one time.

When sections have flattened to approximately the original block size they are collected on pre-numbered microscope slides. Excess water is then drained from the slide before placing in a section-drying oven.

### Haematoxylin and Eosin (H&E) staining of sections.

The normal staining method used to demonstrate general morphology of tissues is the Haematoxylin and Eosin (H&E) method. Diagnosis of TSE disease in rodents is also performed on H&E stained sections.

The procedures for staining and coverslipping of histological sections using an automated staining machine or using the hand staining set are as follows.

The reagents are: Xylene, Industrial methylated spirits (IMS), Haematoxylin, Eosin, Scotts Tap Water Substitute – bluing agent made up as follows:

Chemical	Amount
Sodium hydrogen carbonate	8.75g
Magnesium sulphate	50g
Tap water	2500ml tap water

Pertex mounting medium is also required.

Coverslips and mounting medium are applied to stained slides to protect the sections, increase the refractive index and allow examination by microscopy. Currently and for this project a Leica XL staining machine is used according to manufacturer's instructions. Racks of slides are loaded into the staining machine and stained using the appropriate program. Although Haematoxylin and Eosin are stable solutions giving highly reproducible results test sections are regularly stained for comparison with previous sections. The following is an example of a staining programme although times may vary depending on batches/brands of Haematoxylin and Eosin in use.

Step	Station	Solution	Time in minutes	Exact time
1	1	Xylene	5	
2	2	Xylene	5	
3	3	Xylene	5	
4	4	99%IMS	2	
5	5	99%IMS	2	
6	6	95% IMS	1	
7	Wash 1	Running water	5	
8	7	Haematoxylin	3	yes
9	Wash 2	Running water	3	
10	Wash 3	Running water	3	
11	8	STWS	2	
12	Wash 3	Running water	5	
13	9	Eosin	1.5	yes
14	Wash 4	Running water	30 seconds	yes
15	10	70% IMS	30 seconds	yes
16	11	95% IMS	30 seconds	yes
17	12	95% IMS	30 seconds	yes
18	13	99% IMS	1	yes
19	14	99% IMS	1	
20	15	Xylene/99%IMS	1	
21	16	Xylene	1	
22	17	Xylene	1	

23	18	Xylene	1 (exit drawer)	
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To add the coverslips, Pertex mounting solution is used with an automated Covertex Coverslipper according to manufacturer's instructions.

### Diagnosis of disease.

#### Vacuolation scoring and lesion profiles

Vacuolation scoring is not carried out for sheep in our lab as it is unreliable for some forms of scrapie (eg SSBP/1) which produce little or no vacuolation.

Immunocytochemistry is carried out as a routine on all sheep deaths.

For mouse brains, the scoring is carried out by an expert operative and procedures are in place to ensure uniformity of judgment from different operatives. Nine grey matter areas & 3 white matter areas of the brain (see Appendix 2b) are examined microscopically and given a 'score' from 0-5 (grey) or 0-3 (white) depending on severity of vacuolation of the tissue. A score of zero means no vacuolation (negative) and the scores 1-5 indicate increasing levels of vacuolation. Examples of the scores for grey matter (0 – 5) are shown in Appendix 2c.

Because in mice there is always a certain amount of variation between individuals, at least 5 mice, preferably more, are needed to generate a reliable score for each brain area. To reach the final score, the average of the score for each area is calculated. The average scores are graphed and the resulting shape of the curve is known as a lesion profile which can be very characteristic of particular scrapie strains in mice. An example of a lesion profile for ME7 scrapie in C57Bl mice is shown in Appendix 2e.

For some strains of TSE particularly large deposits of PrP<sup>Sc</sup> protein can be seen in H&E sections as plaques, an example of which is given in Appendix 2e. These will be seen as very darkly staining structures on immunohistochemistry slides if an appropriate anti-PrP antibody is used.

### Immunohistochemistry with BG4 for Tg338 mice

As tg 338 mice express the sheep PrP protein (VRQ allele) on a null background, the antibody BG4 (normally used in this lab for sheep tissues) was used on tissue sections from tg 338 mice. This method had to be developed and optimised first using proven atypical scrapie clinical mice as controls.

#### **PBS/BSA for washes**

250 mls 10xPBS  
5g BSA  
2.5l dH<sub>2</sub>O

#### **PBS/BSA for making up antibodies**

1g BSA  
10mls PBS X 10  
90mls dH<sub>2</sub>O

#### **Methanol with Hydrogen peroxide**

242mls methanol  
8mls hydrogen peroxide

#### **Trypsin**

5.96g tryzma buffer (pH7.6)  
0.2g trypsin  
200mls dH<sub>2</sub>O

### Method

1. Clear paraffin sections in xylene/alcohol/water.
2. Hydrated autoclaving. 121°C for 15 minutes.
3. After cycle complete allow slides to cool for a minute in cold running water then place in PBS/BSA buffer.
4. Place slides in methanol with 1% hydrogen peroxide for 10 minutes.
5. Wash in tap water for 1 minute.
6. Place slides in 0.1% trypsin for 20 minutes.



7. Wash in PBS/BSA buffer.
8. Apply normal rabbit serum 1/20 for 30 minutes. (100µl per slide – 1ml will cover 10 slides).
9. No wash tap of excess and wipe carefully round edges.
10. Apply primary antibody at required dilution. (We would normally use BG4 100µl/section 1:300 dilution) leave overnight on bench. Also apply the normal serum to control section (we normally use normal mouse serum 1:10,000 and leave overnight).
11. Wash in PBS/BSA.
12. Apply biotinylated secondary antibody. We use Rabbit anti mouse dilution 1/500. Leave for 60 minutes.
13. Wash in PBS/BSA.
14. Vector Elite Peroxidase ABC. Use 10µl of each A and B per 1ml. Apply to slides and leave for 30 minutes.
15. Wash in PBS/BSA.
16. Vector red 4 minutes. 5ml distilled water with 3 drops from bottle 1 and 2 drops from each of bottles 2, 3 and 4. Mix between each addition.
17. Put into buffer.
18. Place in Haematoxylin about 1 minute.
19. Place in Scott's tap water for about 1 minute.
20. Place in distilled water.
21. Through from 70% alcohol to xylene keeping time in each short so not to lose staining. And coverslip slides.

### Immunohistochemistry with BG4 for sheep tissues.

This method is our standard process for diagnosis of a TSE in sheep. The anti-PrP monoclonal antibody BG4 (of mouse origin, epitope 46-54, N terminus) is used (Jeffrey et al, 2006). In our hands BG4 used on sheep tissues detects extracellular PrP.

#### **PBS/BSA for washes**

250 mls 10xPBS  
5g BSA  
2.5l dH<sub>2</sub>O

#### **PBS/BSA for making up antibodies**

1g BSA  
10mls PBS X 10  
90mls dH<sub>2</sub>O

#### **Methanol with Hydrogen peroxide**

242mls methanol  
8mls hydrogen peroxide

#### **Trypsin**

5.96g tryzma buffer (pH7.6)  
0.2g trypsin  
200mls dH<sub>2</sub>O

### Method

1. Clear paraffin sections in xylene/alcohol/water.
2. Hydrated autoclaving. 121°C for 15 minutes.
3. After cycle complete allow slides to cool for a minute in cold running water then place in PBS/BSA buffer.
4. Place slides in methanol with 1% hydrogen peroxide for 10 minutes.
5. Wash in tap water for 1 minute.
6. Place slides in 0.1% trypsin for 20 minutes.
7. Wash in PBS/BSA buffer.
8. Apply normal rabbit serum 1/20 for 15 minutes. (100µl per slide – 1ml will cover 10 slides).
9. No wash tap of excess and wipe carefully round edges.

10. Apply primary antibody at required dilution. (We would normally use BG4 100µl/section 1:100 dilution) leave overnight on bench. Also apply the normal serum to control section (we would normally use normal mouse serum 1:10,000 and leave overnight).
11. Wash in PBS/BSA.
12. Apply biotinylated secondary antibody. Use Rabbit anti mouse dilution 1/200 and leave for 60 minutes.
13. Wash in PBS/BSA.
14. Streptavidin Peroxidase Complex (HRP). 10µl of each A and B per 1ml. So if you have 30 slides make up 3mls PBS/BSA with 30µl of A and 30µl of B give a good mix and prepare this mixture 30 minutes before use. Apply to slides and leave for 30 minutes.
15. Wash in PBS/BSA.
16. Vector red 8-10 minutes. 5ml distilled water with 3 drops from bottle 1 and 2 drops from each of bottles 2, 3 and 4. Mix between each addition.
17. Put into buffer.
18. Place in Haematoxylin about 1 minute.
19. Place in Scott's tap water for about 1 minute.
20. Place in distilled water.
21. Through from 70% alcohol to xylene with the time in each kept short so not to lose staining. And coverslip slides.

#### TSE diagnosis in sheep

Diagnosis of a TSE in sheep in this lab is made by examination of immunohistochemistry slides of tissues, brain and/or peripheral, stained with an appropriate anti-PrP antibody. The scoring is done blind so that the operator does not know which sheep he/she is looking at until the coding is broken by the PI later in the analysis process. As this is a routine procedure, sheep are tested as they come

in, rather than waiting for a whole experiment to be finished and doing them all together. The data is mixed up with results from other studies and has to be extracted by the PI from the files and transferred to a storage file, usually Excel.

### Storage of tissues

Fixed tissues are processed to wax blocks and the remains discarded. Blocks and frozen (and sterile frozen) tissues in our archive will be stored for at least 10 years after the end of the experiment, following the Joint Funders' recommendations.

Tissues will be available if needed for future studies and FSA will be consulted prior to their use and prior to disposal as appropriate.

### Experimental outline

Sheep of AHQ/AHQ and AHQ/ARQ (n=4 of each) were to be challenged with atypical scrapie as a 10% brain homogenate taken from 51x45, a proven and clinical case of atypical scrapie. There were 3 serial kill groups, each with the two genotype groups of 4 sheep plus two unchallenged age matched controls for each genotype. Sheep were culled in groups at 6 months following challenge, 12 months following challenge and a final group which would either be clinically affected with atypical scrapie or would be culled at 36 months if still surviving.

All sheep were to have tissues taken and stored for further analysis which would include testing for disease-related PrP protein. In addition, as bioassay is more sensitive than protein analysis at this level, transmissions to tg 338 mice were to be carried out from four clinically affected sheep and if no clinical cases developed, from sheep of the 36 month group as these were judged to be most likely to have detectable levels of infection.

In the event there were no clinical cases in the sheep and so from two AHQ/AHQ and two AHQ/ARQ animals, transmissions to tg 338 mice were set up to detect any infectivity present in brain, spleen, liver and longissimus dorsi (back muscle) taken from the 36 month survivors.

## Results

### Sheep challenges.

Matings were set up between NPU Cheviot sheep of genotypes likely to produce AHQ/AHQ and AHQ/ARQ offspring. The pool of potential parents was relatively small so it took three rounds of lambing to complete the numbers needed for the study; 2007, 2008 and 2009. Because of this, the first group to be set up in 2007 was that requiring up to 3 years observation (36 months), the 12 month group was set up in 2008 and the shortest group (6m) in 2009.

Controls were all concurrent except for one AHQ/AHQ animal in the 36 month group. Due to the unexpected death of the lamb assigned to the project it was necessary to wait until 2009 to assign a new animal to the project. This did not affect the overall project plan.

The potential lambs each year were genotyped at the PrP gene locus, codons 136, 154 and 171, from blood samples taken soon after birth. Once genotypes were assigned, the lambs were dosed with atypical scrapie brain at ages of 36-52 days. The lambs were all returned to their mothers and weaned naturally before being penned separately in mixed groups.

None of the sheep developed signs of a TSE, or signs similar to those observed in the sheep (51x45) that provided the inoculum. One animal died suddenly with no clinical signs of any illness at 125 days of age (12 m serial kill group).

The survival times of all sheep in the study are presented in Table 1 for each of the serial kill groups.

### Pathology on dosed sheep

All sheep in the study were tested by immunohistochemistry (IHC) for signs of disease related PrP (PrPD) in brain and other tissues. We used out standard

method which is known from our previous studies to work well on sheep and does also work well with cases of atypical scrapie we have had in the NPU flock. Areas which are tested from sheep brain are shown in Appendix 3. The anti-PrP antibody BG4 works well on clinical atypical scrapie sheep tissues in our hands.

All sheep in the 6m, 12m and 36 m serial kill groups and controls were negative for PrPD in all tested tissues. An example of the scoring sheets used at the point of examination of the stained sections is given in Appendix 3. The information is transferred to a large Excel file in the order the samples were tested, which means in the Excel file they are mixed in with all the other sheep projects (Defra and BBSRC funded) which are part of the PI's work.

There is often, with sheep, slight hints of non-specific staining, possibly of PrPC, which are not diagnostic of a TSE in our experience. These features have been noted however in Table 1. Examples of negative staining sheep brain are given in Appendix 5, along with controls (both atypical scrapie and classical scrapie) to show what a positive would look like. There are 8 brain sections and a similar number of peripheral tissues for each sheep in this study. We do not routinely photograph each image as the images are very large and data storage must be paid for at Roslin. However the slides are available and also wax blocks to repeat the analysis should this be required and costed.

Since the samples were negative at IHC, it was considered pointless to carry out Biorad extractions for these tissues or to carry out Western blotting. IHC in our hands is by far the most sensitive of these methods for detection of PrPD. The consumables money not used here was used instead to carry out some extra analysis of the bioassay mice, see later.

#### Fate of the parents of the lambs.

Due to the method of dosing of very young pre-weaned lambs, the ewes (dams) may have been exposed to excreted inoculum after the dosed lambs were returned to them. None of the ewes has shown clinical signs of any form of TSE. All lived to 7-9 years of age. The rams, although having no contact at all with their offspring, also lived till older ages without signs of TSE, apart from one young ram which had to be

culled at age 16 months due to intercurrent illness. As is our standard practice, tissues have been taken from the parents for routine pathology however this is still in process so there are no results yet to report, however when the results are in, we will inform FSA.

#### Bioassay in tg 338 mice

Depending on the mouse model used and the strain being detected, bioassay is by far the most sensitive method of detection of infectivity. We therefore carried out inoculations in tg 338 mice from four sheep in the 36 month group, two each of AHQ/AHQ and AHQ/ARQ genotypes. Four tissues from each sheep were chosen. Brain (standard which will allow comparison with previous transmissions), spleen (peripheral tissue which frequently harbours infection even when the brain is negative), liver and longissimus dorsi muscle (both representative of tissues which would be expected to enter the food chain for humans).

After being screened for bacterial contamination, the 10% homogenates all passed the test and were injected into tg338 mice.

#### Tg338 mice observation period.

Incubation period (in our lab) of a bioassay in tg 338 mice from clinical atypical sheep brain is 170-190 days. In another Defra funded study we bioassayed a brain sample from non-clinical atypical scrapie and got an incubation period of 318 days (unpublished). Based on this data, we originally planned to observe any surviving mice for 365 days following challenge. However in February 2011 a paper was published concerning transmissions to tg 338 mice from clinical and preclinical, natural and experimental atypical scrapie infected sheep (Andreoletti et al [2011] Atypical/Nor98 scrapie infectivity in sheep peripheral tissues. Plos Pathogens 7(2): e1001 285.doi:10.1371/journal.ppat.100128S). The sheep in the Andreoletti study were low in number and of the following genotypes: Natural infection> 2 clinicals ( AFRQ/AFRQ and AHQ/AHQ), 3 fallen stock ( ALRQ/ARR; AFRQ/VRQ and ARR/ARR) and 2 found at slaughter (AHQ/AHQ and ARR/ARR). Experimental infection> 2 intracerebral ( AFRQ/ARQ and AHQ/AHQ). It can be seen from this that our current study will add considerably to the knowledge generated by the very

limited bioassays above. However Andreoletti et al reported very long incubation periods from peripheral tissue transmissions, for example 401 days for popliteal lymph node and 450 days for muscle. Many of these tissues were negative in ELISA, Western blotting and IHC but had sufficiently high levels of infection to transmit atypical scrapie to the bioassay mice. As the Andreoletti samples were mostly from clinical sheep, in order for our study of pre-clinical sheep to be both publishable and to be completely reliable, especially if negative, the mouse observation period for the current study was extended, in consultation with FSA, to more than 600 days.

### Mouse transmission results

Experiments were numbered 976A to 979A. Each experiment has a suffix indicating the tissue so that 976A-1B is from brain, 976A-1DM is from muscle, 976A-1L is from liver and 976A-1S is from spleen etc. There were no clinical TSE-like cases in the inoculated tg 338 mice. The survival times of the mice in each tissue/sheep group are given in Appendix 6 A and B. Note that in three of the AHQ/ARQ groups, 12 mice were challenged instead of 6. This nevertheless increases the power of the analysis. For comparison a lifetime survival curve for uninoculated tg 338 mice is given in Appendix 6C – from a project funded by BBSRC.

### Mouse vacuolation (H&E)

Each mouse was examined for vacuolation. Most animals exhibited no vacuolation however there was one animal judged to be showing some low level vacuolation (mouse 5 from 976A-1S) (see Appendix 7 for examples of the pathology scores for this study). This transmission was from spleen of an AHQ/ARQ sheep. Another animal (mouse 1 from 977A-1L) had a possible plaque and other animals had inconclusive vacuolation (this usually the result simply of ageing). None of the mice in the ageing experiment Appendix 6C scored positive for TSE-like vacuolation. An example of a lesion profile from clinically affected tg 338 mice with atypical scrapie is shown in Appendix 1 and some examples of H&E sections from the positive scoring mouse and a negative scoring mouse are given in Appendix 8. It should be noted that these pictures are jpeg files transformed from tif files in order to avoid placing



enormous files in this report. Each tif file representing one small area of tissue has a size of at least 14Mb. The quality of the photos has suffered as a result of being saved as jpegs but Appendix 8 gives an indication of the appearance of the results. We do not routinely photograph mouse vacuolation or scoring however tissue blocks and slides are kept for at least 10 years following the end of the experiment and would be available for future studies.

### Mouse immunohistochemistry

Andreoletti et al (2012) noted in their atypical scrapie transmissions to tg 338 mice, a number of animals which were positive in immunohistochemistry (IHC), for detection of PrPD, when they were negative by other tests such as Western blot. Because of this we carried out IHC on the tg 338 mice from this study.

This was the first time we had tried IHC with tg 338 mice in any circumstances so a new method had to be developed and first proven to work on known clinical atypical scrapie infected mice. Various antibodies were tested including BG4, R145 and P4. First attempts were negative, even in the positive controls so it was necessary to change and then optimise the method, which took some time but was however successful and is detailed in the methods section above. An example of positive staining for PrPD in tg 338 mice clinically positive for atypical scrapie is given in Appendix 9. This photo shows a characteristic feature of clinical atypical scrapie in tg 338 mice, namely a double banded pattern in the cerebellum. This banding is not detected in tg 338 mice infected with classical scrapie, in our hands and an example of classical scrapie in tg 338s is shown for comparison in Appendix 9.

IHC results for the mice in this study were unusual and mostly showing positive staining. Since this was not what we expected, we also tested the uninoculated tg 338 mice in the ageing study (BBSRC funded). These animals also showed some staining with BG4. Examples of the staining from challenged mice are shown in Appendix 10.

### Discussion

In this project we carried out an experimental oral infection of AHQ/AHQ and AHQ/ARQ sheep with atypical scrapie. Within the timespan of the project we had no clinical cases in the sheep however it was possible that within their body tissues atypical scrapie could have been present at low levels following ingestion. The chances of successful oral infection were optimal as young lambs before weaning are highly susceptible to TSE infection (Hunter et al 2012) however other studies have also found that it is difficult to infect sheep with atypical scrapie, although not impossible (Simmons et al 2009 and 2011).

Tissues from three serial kill points (6 m, 12m and 36 m) were examined by immunohistochemistry for signs of PrPD without success. However as bioassay in mice is a more sensitive method for detection of infectivity, bioassays were carried out into tg 338 mice from brain, spleen, liver and muscle tissues from four sheep – two AHQ/AHQ and two AHQ/ARQ animals. Again there were no clinical atypical cases in the mice and very little vacuolation pathology, most mice were negative for vacuolation although at least two showed low level pathology.

In order to confirm (or otherwise) the vacuolation results, brains from all the mice in the bioassays were tested by immunohistochemistry using the antibody BG4 to detect any disease related PrP that might be present. Although not carried out routinely in mice, it is sometimes the case that pre-clinical animals can show signs of PrPSc deposition prior to vacuolation occurring. In addition Andreoletti et al (2011) found that tg 338 mice infected with atypical scrapie could be immunohistochemistry positive when Western blots and ELISA were both negative. Our study found that virtually all of the mice were showing signs of staining with BG4. As this was considered surprising, we also tested uninoculated tg 338 mice which had been controls in another experiment (BBSRC funded) and had been allowed to age without infection. These mice also showed staining with BG4 suggesting the detection of PrPD was at least partly related to the transgenic mouse line and not to infection with atypical scrapie.

In a recent study (Gotte et al 2012) it was noted that uninoculated tg 338 mice in their experiments had not only low level vacuolation but also were positive at low levels on immunohistochemistry. We have never seen this phenomenon in previous

experiments which have been “clean” which means that negative uninoculated controls were pathology negative but it remains possible that the same issue as observed by Gotte et al (2012) has affected the mice in the present study.

Gotte et al (2012) have also reported a method to distinguish between the endogenous PrP-positive signal in tg 338 mice and that which is truly indicative of an infection. This involves carrying out Western blots in a particular way that requires considerable skill and expertise. However Western blotting of uninoculated tg 338 mouse brain revealed faint signs of PrP protein with the characteristic low molecular weight band associated with atypical scrapie. Running the gels for the Western blots very carefully it was apparent that the band was actually a doublet of approximately 5kD and 8kD. Treatment of the protein with increasing amounts of proteinase K showed that the 8kD band is much more resistant and the 5kD band disappears at lower proteinase K concentrations. The authors think the 8kD band is coming from the tg 338 mice themselves and could in theory be the source of the deposits of positive staining we have seen in our mice.

All of this needs to be confirmed in our mice. It is not possible yet to dismiss all the positive PrPD staining in the inoculated tg 338 mice without carrying out further studies. It may be possible to use other antibodies which may bind to the PrP molecule in a region outwith the 8kD fragment and/or we must develop the Gotte Western blot method and to find out if our mice are producing the same results. We hope to be able to carry out this work during the coming 6 months.

## Conclusions

Our results suggest that firstly it is difficult to transmit atypical scrapie to sheep, even with the susceptible genotype. Non-clinical challenged sheep could still be infected in peripheral tissues, or even in brain, however immunohistochemistry and bioassays in very sensitive mice did not show any signs of infection clinical infection. Close examination of the mice suggested a very low level of infection might be present in some sheep. However unexpected and potentially confounding factors with the transgenic mice mean further work is needed to be sure of the results. The

indicators of infection are all suggesting low to negligible levels are present in our sheep but the detail of this must await further study.

Even if the IHC results are correct, for this very sensitive mouse not to show clinical signs in under 200days but instead survive for over 600 days still means that only very low levels of infection could be present. We expect to be able to confirm that known atypical scrapie infected sheep, at preclinical stages, harbour low to negligible amounts of infectivity in brain, spleen, liver and muscle tissues.