FINAL REPORT

Quantification of PrPd and correlation with infectivity in scrapie infected sheep tissues destined for human consumption

MO3060
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Animal Health and Veterinary Laboratories Agency

The Secretary of State for Environment, Food and Rural Affairs
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1. Background

Scrapie is endemic in the sheep flocks of Great Britain and the causative agent of this progressive, neurodegenerative disease of the central nervous system is a prion. Prions are chromosomally-encoded proteins that exist as conformational isomers of one of two (or several) covalently identical forms, and the prion form transmits disease by inducing the other (normal or cellular form) to a new copy of the prion form. In mammals, scrapie in sheep, Creutzfeldt-Jakob disease (CJD) of humans and bovine spongiform encephalopathy of cattle (*inter alia*) are caused by the prion form (PrP^{Sc}) of the cellular prion protein (PrP^{C}) of their respective species. Tragically, the prions of one species can infect other species and some are zoonotic agents (for example, the BSE prion of cattle can cause a variant form of CJD).

To protect humans from BSE, cattle tissues which may contain significant amounts of prions are removed and destroyed to prevent them entering into the food chain. These specified risk materials (SRM) included brain, spinal cord and various parts of the gut and associated lymphoid tissue. The prions of scrapie are not thought to be zoonotic agents but, in accord with the precautionary principle, sheep SRMs are similarly removed from carcases to prevent undue exposure of humans; this is partly due to a (diminishing) risk that BSE prions might have replicated in the GB sheep population and partly in acknowledgement that the diversity (and zoonotic potential) of all types or strains of sheep prions is unknown. The removal of SRM from sheep is theoretically less effective at reducing exposure to humans than identical measures applied to the cattle population (if the prevalence of infection were the same) because of the relatively wide dissemination of these infectious agents in sheep carcases compared to their distribution in cattle.

The effectiveness of removal of ovine SRM at reducing risk of exposure to prions at a European level is under continual review because of the expense and inconvenience it creates for the farmer, butcher, veterinarian and chef but a proportionate perspective of the issue is difficult because of the lack of quantitative data on the infectious load in various edible tissues of the sheep. Historically, quantal titration in mice by intracerebral challenge with tissue extracts was the only way a measure of infectious titre of these agents could be estimated, and most semi-quantitative assessments of exposure rely on a single study of titrating tissues from Suffolk sheep in wild-type (with respect to the prion protein gene) laboratory mice by Hadlow and colleagues for their input data of infectious load [1].

Operationally, PrP^{Sc} can be distinguished from PrP^{C} in the laboratory by its differential detergent solubility and by its relative resistance to proteolysis under mild, denaturing conditions. Early work by McKinley and Prusiner, and Diringer, in the 1980's established a stoichiometry of ~ 100 000 PrP-res (the proteinase-resistant core of PrP^{Sc}) molecules for each infectious unit of prion infectivity and, although fractions of disease-related abnormal prion proteins have been reported with a more variable specific infectivity, the use of a biochemical measurement of PrP^{Sc} (or PrP-res) to infer titre has always offered a powerful opportunity to risk managers seeking a balanced view of infectious load in

exposure risks. The development of commercial, EU-validated rapid biochemical tests for abnormal PrP and the introduction of relatively quick transgenic mouse bioassay models in 2003 re-stimulated this strategy to improve our quantitative perspective of human exposure to sheep prions.

This study at AHVLA, and a parallel investigation at the Moredun Research Institute (MRI) (MO3059), was commissioned by the Food Standards Agency to provide proof-of-principle data on the correlation between infectivity and biochemical measurements in edible tissues from Suffolk sheep of the common ARQ/ARQ prion protein genotype in a naturally-infected scrapie flock at MRI.

2. Objective 1 : Quantify PrP^d in edible tissues derived from sheep infected with scrapie.

2.1 Choice of flock and choice of tissues

The Suffolk flock at MRI has been extensively studied since the 1980s and the dynamics and spread of its prions in peripheral tissues and their transmission characteristics are well documented [2; 3]. From this flock, we necropsied six sheep of the ARQ/ARQ PrP genotype between the age of 22 and 28 months which were naturally affected with clinical scrapie. A full post-mortem dissection was carried out on each sheep and a wide range of tissues were blocked and processed through paraffin wax for histological examination, or frozen at -20C for biochemical analysis. An identical range of tissues from two control animals were also prepared.

Selected tissues were stained with haematoxylin and eosin and by immune-histochemistry (IHC) for abnormal PrP (PrP^D) using the R145 antibody (AHVLA). Disease was confirmed in the clinical scrapie cases by finding vacuolation of the brain neuropil and abnormal PrP IHC (termed PrP^D) staining. The intensity and distribution of PrP^D in the brain was subjectively scored as previously described [4; 5]. PrP^D was also present in peripheral autonomic system tissues, in lymphoid tissues, retina, adrenal medulla, in spindles of muscles and in kidney. However, PrP^D was not detected in myocardium or in the sensory taste receptor or nerves of the tongue. In muscle, PrP^D was confined to muscle spindles and was not detected in myocytes. PrP^D was detected in only one sample of semi-tendinosis muscle but was detected in all samples of ocular muscle. This reflects the greater density of spindles in ocular muscles. Whereas muscle spindle samples were detected in histological sections from semi-tendinosis muscle from only one sheep, numerous (n>5) spindles were detected in all ocular muscle samples from all scrapie-affected sheep and controls. Multiple samples (n>3) of renal pelvis were sampled from each sheep. Only two sheep showed PrP^D accumulation in renal pelvis tissues. No PrP^D was detected in any tissue sample taken from controls.

Based on our pathological findings, tissues from the most severely-affected sheep were selected for further biochemical analysis and bioassay: brain, ocular muscle, tongue, pre-scapular lymph node, heart, kidney, liver, sciatic nerve and semi-tendinosis muscle.

Biochemical Analysis : Methods

Two commercial kits for the detection of abnormal PrP were adapted for use in this study : the IDEXX HerdCheck CWD Antigen EIA test and the Bio-Rad TeSeE ELISA. Both tests had previously undergone an EU-evaluation of their diagnostic sensitivity and specificity on ruminant brain stem (and some peripheral tissues)(IRMM, 2005, quoted in [6]), and their analytical sensitivities have recently been reported [6].

While both tests use a detection antibody raised against part of the prion protein (and coupled to a reporter molecule), one test uses a polyvalent ionic ligand to capture analyte in a microtitre plate well format without prior digestion with proteases (IDEXX), while the other uses detergent extraction, proteinase K hydrolysis and a second anti-PrP monoclonal antibody immobilised to the microtitre well as capture reagent (Bio-Rad TeSeE).

We modified the manufacturers' protocols for both assays and used tissues from VRQ/VRQ sheep in the SE230 naturally-infected flock at AHVLA Weybridge to optimise our procedures. The results of our application of these tests to the target MRI Suffolk flock are presented here.

a) IDEXX HerdCheck CWD Antigen EIA test

For the determination of PrP^{D} *in the absence of PK,* the "IDEXX HerdChek CWD Antigen EIA Test" was carried out according to the manufacturer's instructions with some modifications. In brief, the samples were homogenised as described but with the incorporation of a single large (6mm diameter) ceramic bead and by performing three agitation cycles at 6.5rpm each for 45 seconds; the homogenates were cooled between each cycle. This facilitated better homogenisation of tissues less readily disrupted than brain. The samples were mixed with the working plate diluents, loaded on to the antigen-capture plate and shaken for 150 minutes at room temperature. PrP^{sc} was observed using the kit-conjugated anti-PrP antibodies, visualised with a colorimetric substarte and absorbance read at 450nm and 620nm. For end-point dilution, the samples were serially diluted in normal brain homogenate prior application to the plate and treatment with working plate diluent.

b) The Bio-Rad TeSeE BSE ELISA

This ELISA is approved for use for the diagnosis of BSE in cattle and scrapie in small ruminants. The test was used as directed by the kit insert provided by Bio-Rad Inc. However, a modified protocol was developed in order to investigate digestion of PrP at low concentrations of PK in the various non-neural tissue matrices. The tissue was ribolysed using a single large (6mm diameter) ceramic bead, to give 20% (w/v) homogenate. DNAse was incorporated prior to the PK treatment and Pefabloc^{TM} was added in addition to the kit protease inhibition solution. The PK concentration was titrated from a Sigma^{TM} stock solution. Following precipitation and centrifugation at 15,000 g for 7 minutes, the pellets were solubilised by incubating at 100^{o}C for 5 minutes in kit Reagent C. Thereafter the method was followed as directed by the manufacturer. The absorbance was measured at 450nm and 620nm.

To determine the lowest concentration of PK appropriate to each tissue, samples from unexposed control sheep were exposed to different concentrations of PK. To determine the relative concentrations of PrP^{C} in each tissue, we also assayed for PrP content in the absence of PK. The relative undigested PrP amounts observed correlated with those published by Moudjou et al [7] and the range of PK concentrations required to digest all PrP^{c} ranged from 0.086 units/ml (prescapular lymph node) to 1.72 units/ml (tongue, kidney and semitendinosus muscle). When spiked with scrapie positive brainstem and digested using the pre-determined concentration of PK, each matrix (n=2) gave OD readings above the designated assay cut-off point and the matrix did not significantly interfere with the detection of PrP^{D}.

The level of PK selected for each tissue for use in the adapted Bio-Rad TeSeE ELISA is detailed in Table 1.

Table 1

	PK (units/ml)
Brain	6.864
Ocular Muscle	0.858
Tongue	1.720
Prescapular Lymph Node	0.086
Heart - Left Ventricle	0.858
Liver	0.343
Kidney	1.720
Sciatic nerve	0.343
Semitendinosus Muscle	1.720

Table 1: *The lowest concentration of PK at which all PrP^{c} was digested, as determined using PK titration curves, for each tissue. Each assay was performed in duplicate.*

To determine relative quantities of PrP^{D}, samples were diluted in order to reach a value that coincided with the linear portion of a standard curve of recombinant PrP (rPrP). Relative quantities

were then determined using extrapolation from the curve using Prism version 4 software.Threshold values for each individual tissue were also calculated. Eight tissues from ARQ/ARQ negative control sheep, from a TSE-free flock, were assayed using both modified protocols and the mean optical density of all replicates (n≥15) calculated for each tissue using both assays. The mean optical density value + 3 standard deviations were calculated as the threshold values for all subsequent assays, with a value above these cut-off values considered to be positive.

i) Biochemical Analysis : Qualititive Results and Ranking of Tissues for PrP^D content

Semitendinosus muscle, extraocular muscle, tongue, heart, liver, kidney, prescapular lymph node, sciatic nerve and brain from four scrapie affected ARQ/ARQ Suffolk sheep and one unexposed animal were analysed for PrP^D content using the modified IDEXX Herdchek EIA and the modified Bio-Rad TeSeE ELISA (Table 2).

PrP^{sc} was confirmed in brain, ocular muscle, prescapular lymph node and kidney in all 4 sheep tested, although assays were not always coincident. We have previously shown that the IDEXX assay is more analytically sensitive than the Bio-Rad TeSeE ELISA and this could explain the results observed for the sciatic nerve. It does not, however, explain the observations in other tissues where differences might reflect the differences in the nature of the PrP detected by the two assays. Furthermore, discrepancies might be explained by sampling issues and this is particularly emphasised where tissues are not homogeneously infected e.g. kidney. Three of the four sciatic nerves tested were positive for $PrP^{D.}$ This tissue was the most difficult to process and this could contribute to inconsistent extraction.

Semitendinosus muscle was negative in all cases. PrP is known to accumulate in muscle spindles but in any plane of semitendinosus muscle, $1cm^2$, only 1 in 10 is likely to contain spindles (Jeffrey, unpublished observations). Lymphoid and nervous tissue is retained in association with red meat after removal of ovine SRM and it is likely from this analysis that their presence would contribute more to the infectivity in meat than muscle itself. Ocular muscle contains a large number of spindles known to be PrP^{sc} positive (as observed by IHC) and these are likely to contribute to the high levels of PrP^{sc} observed in this tissue. Significantly, PrP^D was not detected in heart or liver, and it was detected in the tongue in only 1 animal.

Table 2

SHEEP IDENTIFICATION														
	1216B			1223B			1221B			1217B			N523	
	Idexx	B.Rad	IHC	Idexx	B.Rad	IHC	Idexx	B.Rad	IHC	Idexx	B.Rad	IHC	Idexx	B.Rad
Brain	2.770	2.862	3	2.781	2.817	3	2.814	2.773	3	2.793	1.860	3	0.018	0.006
Ocular M	0.451	0.063	3	0.094	0.034	3	0.221	0.006	3	0.227	0.049	3	0.031	0.005

Tongue	0.041	0.025	0	0.044	0.021	0	0.045	0.018	0	0.032	**0.137**	0	0.035	0.013
Prescap. LN	**0.822**	**1.670**	**3**	**2.816**	**1.770**	**3**	**2.598**	**1.403**	**3**	**1.892**	**2.630**	**3**	0.045	0.004
Heart - Left vent	0.061	0.007	0	0.049	0.014	0	0.042	0.021	0	0.039	0.013	0	0.038	0.008
Liver	0.038	0.006	0	0.031	0.005	0	0.034	0.006	0	0.042	0.006	0	0.027	0.006
L. kidney	0.044	**0.223**	0	**1.420**	**0.048**	**3**	0.050	**0.045**	0	0.047	**0.020**	**2**	0.030	0.016
R. kidney	0.048	**0.095**		**0.131**	**0.028**		0.042	nd		0.059	**0.020**		0.063	0.015
Sciatic nerve	**0.333**	0.009	**1**	**0.538**	0.007	**1**	0.049	0.011	**1**	**0.438**	0.008	**2**	0.041	0.002
L. semi-tend M	0.054	0.022	**2**	0.058	0.015	0	0.050	0.027	0	0.053	0.014	0	0.044	0.015
R. semi-tend M	0.050	0.024		0.060	0.015		0.044	nd		0.050	0.015		0.045	0.011

Table 2: *Detection of* PrP^{sc} *in target tissues analysed from 4 scrapie infected, MRI sourced, Suffolk sheep (1216B, 1223B, 1221B, 1217B) and a single non-exposed sheep (N523) using the modified Bio-Rad and IDEXX protocols and IHC analysis. Results recorded in red exceed the tissue-specific assay cut-off values. Tissues from the sheep underlined were submitted for bioassay in both bank voles (MRI, SE3059) and TgsheepXI mice. Values for the Bio-Rad and IDEXX tests are absorbance values (450-620 nm).* The intensity and distribution of PrP^{D} detected by immunocytochemistry in the brain was subjectively scored on a scale 0 (little or no IHC staining) to 3 (intense IHC staining) as previously described [4; 5].

Table 3 below presents the same data transformed into a relative score to allow a ranking comparision of the biochemical tests with the subjective immune-histochemical score previously developed by AHVLA pathologists [4; 5] No tissues from the control animal exceed the pre-determined cut-off thresholds emphasising the specificity of these tests.

Table 3

SHEEP IDENTIFICATION														
	1216B			**1223B**			**1221B**			**1217B**			**N523**	
Tissue	**Idexx**	**B.Rad**	**IHC**	**Idexx**	**B.Rad**	**IHC**	**Idexx**	**B.Rad**	**IHC**	**Idexx**	**B.Rad**	**IHC**	**Idexx**	**B.Rad**

Brain	+++ +	+++ +	++ +	+++ +	+++ +	++ +	+++ +	+++ +	++ +	+++ +	+++ +	++ +	-	-
Ocular M	+	+	++ +	-	+	++ +	+	-	++ +	+	+	++ +	-	-
Tongue	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Prescap. LN	++	+++ +	++ +	+++ +	+++ +	++ +	+++ +	+++	++ +	+++ +	+++ +	++ +	-	-
Heart - Left vent	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. kidney	-	+	-	+++	+	++ +	-	+	-	-	+	++	-	-
R. kidney	-	+		+	+		-	nd		-	+		-	-
Sciatic nerve	+	-	+	++	-	+	-	-	+	+	-	++	-	-
L. semi-tend M	-	-	++	-	-	-	-	-	-	-	-	-	-	-
R. semi-tend M	-	-		-	-		-	nd		-	-		-	-

Table 3: *Summary of PrP^D detection in edible tissues by immunochemical assay. The number of +'s indicates strength of signal. L= left and R= right tissues sampled. M=muscle. Shaded rows indicate no PrP^D was detected in these tissues by immunoassay.*

ii) Biochemical Analysis : Quantitative Results and Ranking of Tissues for PrP^D content

In principle, the IDEXX HerdChek assay relies on an aggregated state of the PrP analyte to allow efficient capture in the first phase of its protocol. This excludes the use of soluble recombinant prion protein as a standard to calibrate the assay signal (absorbance units) in terms of a known amount or weight of prion protein. However, to provide a more objective estimate of the relative levels of abnormal prion protein in brain and peripheral tissues from scrapie-affected animals, we were able to obtain end-point dilution values for each tissue using the IDEXX HerdChek test (Table 4). For example, the signal from brain was still above the threshold level when sample from all four scrapie cases was diluted by 1000 -2000 times; relative levels in pre-scapular lymph node where lower and more variable, and levels in ocular muscle and sciatic nerve were lower still and in some cases only detected in undiluted tissue extract (ocular muscle) or not at all (tongue, kidney).

Quantities of PrP^{res} relative to a standard curve of rPrP could be obtained for the Bio-Rad TeSeE assay (Table 4). In all cases, the brain contained the highest levels of PrP^{res} at 2 microgram per g of tissue (n =2), and the assay has a dynamic range of ~10^3 which allows quantification of abnormal PrP in kidney at ~ 5 ng/g .

Table 4: Quantification of PrP^{sc} in edible tissues

	1216B	1223B

	IDEXX End pt dilution	BIO-RAD – PrP concn (µg/ml)	IDEXX End pt dilution	BIO-RAD – PrP concn (µg/ml)
Brain	1/2048	2.04	1/1024	2.01
Ocular M	neat	0.084	-	0.064
Tongue	-	-	-	-
Prescap. LN	1/64	1.698	1/512	2.006
L. kidney	-	0.168	1/128	0.026
R. kidney	-	0.062	1/2	0.011
Sciatic nerve	1/8	-	1/8	-

	1221B		1217B	
	IDEXX End pt dilution	BIO-RAD – PrP concn (µg/ml)	IDEXX End pt dilution	BIO-RAD – PrP concn (µg/ml)
Brain	1/2048	1.978	1/1024	1.34
Ocular M	1/4	0.045	1/4	0.075
Tongue	-	-	-	0.094
Prescap. LN	1/32	1.403	1/128	1.878 (L) 0.817 (R)
L. kidney	-	0.024	-	0.005
R. kidney	-	-	-	0.005
Sciatic nerve	-	-	1/16	-

Table 4: *End-point dilutions determined using the IDEXX Herdchek assay and levels of PrP^{sc} calculated from Bio-Rad TeSeE rPrP standard curves.*

Objective 2 : Titrate infectivity in target tissues by bioassay in transgenic mice.

The choice of the MRI Suffolk flock for this study was based on several criteria : i) it is a naturally-infected flock of long standing; ii) the survival time of animals born into the flock is known, iii) the pathogenesis of disease from birth to clinical end-point had been previously investigated using conventional histo-pathological and immune-histological methods, iv) the uniform pathological phenotype in brain and peripheral tissues was consistent with a single source infection and v) and the predominant prion protein genotype of the animals in the flock is ARQ/ARQ, a “susceptible” genotype which is common in the GB national flock. This knowledge, and the conventional three-band PrP^{res} Western blot profile of its brain abnormal prion protein, gave confidence at inception that any information on a correlation between infectivity and biochemical assay of abnormal prion protein would be useful for estimation of infectious load in a quantitative risk assessment.

In their “Protocol for the evaluation of rapid post mortem tests to detect TSE in small ruminants” [8], the BioHazards Panel of EFSA recommended the calibration of biochemical tests by bioassay should

use tg338 mice (over-expressing the ovine VRQ PrP allele) for bioassay of sheep scrapie samples and tg110 (over-expressing the bovine PrP 6-octa repeat allele) for bioassay of samples likely to contain BSE prions. Our intitial attempts to titrate the MRI Suffolk scrapie source in tg 338 mice as part of this project revealed a prolonged incubation time of > 500 days before the onset of clinical signs following challenge with a 10^{-1} dilution of brain homogenate from a clinical case. On consultation with FSA, it was agreed to switch the bioassay model to tgshpXI mice at AHVLA, and provide samples of 1223B and 1216B to Dr Umberto Agrimi, SSI, Rome for inoculation into bank voles (under MO3059); Dr Agrimi had already shown the Suffolk source had similar transmission characteristics in voles to a common Italian scrapie isolate (~ 250 days p.i for a 10-1 dilution of a clinical case brain homogenate). Essentially, this has delayed the completion of this project by two years and only now have all the tgshpXI mice been culled and examined for signs of prion disease. The survival times for animals inoculated with tissues from FSA3B (1223B) and FSA6B (1216B) and the estimated titres of these inocula are provided in Table 5.

TgshpXI mice inoculated with serial dilutions of brain homogenate from the two clinical cases of MRI Suffolk scrapie had a range of survival times from 320-600 days within and between dilution groups although those mice diagnosed within a higher dilution group with prion disease had on average a longer survival time than those in lower dilution groups. Both cases had titres on prions at a level of 10^8 ic LD_{50} units per g of brain tissue. Levels of infectivity exceeding 10^3 ic LD_{50} units per g were found in pre-scapular lymph nodes from both cases, and similar but more variable titres were seen in samples of sciatic nerve and ocular muscle. Consistent low levels of infectivity (~ 10^2 ic LD_{50} units per g) were found in tongue from both sheep sampled, and a similar level was found in kidney from one sheep (1216B) but not the other. No infectivity was detected by this bioassay in heart, liver or semi-tendinosus muscle.

Table 5

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Inoculum code	**Sample ID**	**Tissue**	**Dilution**	**Mouse strain**	**IHC result/days PI**										**Hit rate (%)**	**Titration result mouse i.c LD50/g of tissue**
M03060/0001	FSA3B-a	Kidney	10^{-1}	TgShpX1	300	335	349	363	371	391	399	399	419	437	0	0
M03060/0002	FSA3B-b	Semitendinosus	10^{-1}	TgShpX1	327	384	479	486	583	682	689	689	710	767	0	0
M03060/0003	FSA3B-c	Sciatic nerve	10^{-1}	TgShpX1	258	296	343	504	524	531	531	632	666	735	100	$>10^{3.20}$
M03060/0004	FSA3B-d	Prescapular Lymph node	10^{-1}	TgShpX1	0	148	216	417	431	515	522	538	550	573	100	$>10^{3.20}$
M03060/0005	FSA3B-e	Oculomotor muscle	10^{-1}	TgShpX1	385	417	508	510	524	524	604	636	652	664	100	$>10^{3.20}$
M03060/0006	FSA3B-f	Heart	10^{-1}	TgShpX1	202	466	488	505	623	672	672	672	694	774	0	0
M03060/0007	FSA3B-g	Tongue	10^{-1}	TgShpX1	291	299	308	405	500	508	524	645	699	699	43	$10^{2.13}$
M03060/0008	FSA3B-h	Liver	10^{-1}	TgShpX1	379	405	405	471	471	483	483	483	538	667	10	$10^{1.80}$
M03060/0015	FSA6B-a	Kidney	10^{-1}	TgShpX1	0	143	254	254	412	441	510	596	659	706	75	$10^{2.46}$
M03060/0016	FSA6B-b	Semitendinosus	10^{-1}	TgShpX1	291	405	445	480	488	499	604	664	673	673	0	0
M03060/0017	FSA6B-c	Sciatic nerve	10^{-1}	TgShpX1	266	350	370	405	452	524	542	602	689	776	83	$10^{2.63}$
M03060/0018	FSA6B-d	Prescapular Lymph node	10^{-1}	TgShpX1	253	343	395	433	492	492	527	589	603	671	100	$>10^{3.20}$
M03060/0019	FSA6B-e	Oculomotor muscle	10^{-1}	TgShpX1	311	371	382	396	409	455	594	595	808	808	43	$10^{2.13}$
M03060/0020	FSA6B-f	Heart	10^{-1}	TgShpX1	408	447	454	510	576	646	646	685	804	861	0	0
M03060/0021	FSA6B-g	Tongue	10^{-1}	TgShpX1	406	473	529	671	678	686	686	692	692	733	25	$10^{1.95}$
M03060/0022	FSA6B-h	Liver	10^{-1}	TgShpX1	62	67	71	197	216	327	341	502	502	574	0	0
M03060/0029	FSA1657-h	Liver	10^{-1}	Tg338	363	393	407	440	509	561	577	610	637	719	100	$>10^{3.20}$

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Inoculum code	**Sample ID**	**Tissue**	**Dilution**	**Mouse strain**	**IHC result/days PI**										**Hit rate (%)**	**Titration result mouse i.c LD50/g of tissue**
M03060/0009	FSA3B-i	Brain	10^{-1}	TgShpX1	319	342	358	432	432	489	546	562	578	603	100	$10^{8.05}$
M03060/0010	FSA3B-i	Brain	10^{-2}	TgShpX1	158	313	348	403	466	479	494	578	580	613	100	
M03060/0011	FSA3B-i	Brain	10^{-3}	TgShpX1	267	387	407	509	554	561	575	575	589	658	100	
M03060/0012	FSA3B-i	Brain	10^{-4}	TgShpX1	216	272	279	464	526	554	575	617	678	754	83	
M03060/0013	FSA3B-i	Brain	10^{-5}	TgShpX1	359	589	589	600	610	637	659	698	705	705	25	
M03060/0014	FSA3B-i	Brain	10^{-6}	TgShpX1	429	467	481	488	526	533	533	705	705	705	10	
M03060/0023	FSA6B-i	Brain	10^{-1}	TgShpX1	231	312	385	413	460	502	502	508	508	574	100	$10^{7.70}$
M03060/0024	FSA6B-i	Brain	10^{-2}	TgShpX1	282	377	511	532	539	542	559	566	570	570	100	
M03060/0025	FSA6B-i	Brain	10^{-3}	TgShpX1	356	409	433	458	545	545	580	642	642	685	100	
M03060/0026	FSA6B-i	Brain	10^{-4}	TgShpX1	350	356	364	433	440	507	538	566	566	622	100	
M03060/0027	FSA6B-i	Brain	10^{-5}	TgShpX1	201	341	462	500	500	539	545	545	657	710	0	
M03060/0028	FSA6B-i	Brain	10^{-6}	TgShpX1	118	165	249	263	350	356	364	405	405	465	0	

222	Positive result
222	Inconclusive result
222	Negative result
0	No tissue available

During the preliminary workup of the biochemical assays for peripheral tissues, we used samples from naturally-infected VRQ/VRQ sheep from our endemic flock at AHVLA Weybridge, and BSE challenged ARQ/ARQ sheep, and found abnormal PrP in 8/9 scrapie-affected sheep liver samples and 7/7 BSE in sheep liver samples [9] by both BioRad and Idexx tests. In one VRQ/VRQ animal, we were able to detect abnormal prion protein using the BioRad assay at a level of 55 ng/mL (g). A sample of this liver was also inoculated into tg338 mice and produced a 100% attack rate in this model: this is also reported in Table 5 (see FSA1657-h) and contrasts to the negative bioassay and biochemical test data we obtained from liver tissue sourced from the MRI Suffolk flock.

Conclusions

*Objective 3: Can these data be used to evaluate a correlation between titres of scrapie in transgenic mice with biochemical quantification of PrP^d**?***

The range of 10-fold dilutions of sample which can be made and yet still retain a bioassay or biochemical test value above a negative cut-off threshold is termed its "dynamic range" and for the Suffolk scrapie/tgshpXI model this approximates to 10^8, although the variability in these bioassay measurements can be up to 10-100-fold; for both biochemical tests and Suffolk scrapie the dynamic range is between 10^3 and 10^4 . In both bioassay and biochemical tests this range is defined in terms of dilution of clinical, Suffolk scrapie-affected sheep brain.

The bioassay response is very dependent on the mouse model chosen for assay (for example, the tg338 model was not suitable for assaying MRI Suffolk sheep prions) and similarly different sources of prions (for example, atypical scrapie prions vs Suffolk prions) differ in their response to various commercial BSE/scrapie diagnostics tests; BSE/scrapie diagnostic tests for use in surveillance within the EU are approved if they are no more than 100-fold less sensitive than the "best" test (EFSA, 2007). We share the view stated by EFSA's experts when they drew up the protocol for assessing the analytical sensitivity of PrP-based TSE diagnostic tests and their calibration against bioassay in mice (EFSA, 2007):

"...these [bioassay] data will be used to calibrate the test response to a number of infectious particles, which will allow a comprehensive comparison of test performance with reference to biological (gold) standard. However this specific infectivity of a sample would only have relevance to a particular animal bioassay model, and results should not be used to infer the risk of infection following exposure of humans or animals to that sample."

Considering the biological variability of the bioassay and the poorer biochemical sensitivity of rapid tests compared to bioassay, it is encouraging that there is a good correlation between the level of prions in brain and the high end-point dilution of this tissue in the biochemical assays; and a similar ranking of bioassay titre and tissue end-point dilution value is seen for pre-scapular lymph node, sciatic nerve and ocular muscle although the rate of decline of the biochemical signal at high dilutions seems to be faster than for prion titre itself; in practice this could lead to an under-estimation of infectious load solely based on biochemical assay if this effect is not taken into account. However, this is probably less of a confounding factor for the risk manager to take into account than the diversity of scrapie itself: neither bioassay or biochemical assay found evidence of infection in Suffolk-scrapie-affected sheep liver while it is clear this organ is infected by BSE ARQ/ARQ prions or AHVLA VRQ/VRQ prions (cf Everest et al., 2011 [9], and Table 5, sample FSA1657-h).

Scrapie is a prion protein disease and, in their Opinion on the quantitative risk assessment on the residual BSE risk in sheep meat and meat products[10] the EFSA BIOHAZ Panel considered that "*while absolute quantification of prions by biochemical methods is difficult, and the experiments needed to correlate their outputs to bioassay titres costly and time-consuming, measurements of abnormal PrP in two tissues of the same animal may be compared as a first approach to an assessment of the ratio of infectivity in each tissue, and their intrinsic relative risk following exposure to humans*". Biochemical assays can similarly be used to monitor the timing and relative amounts of infectivity in tissues of TSE affected animals, and a biochemical assay approach to the problem of estimating infectious prion load in sheep tissues featured heavily in the recent EFSA risk assessment on the changes in human exposure to sheep prions that might result from different age-related, ovine SRM scenarios (EFSA, 2010). This MO3060 study adds information to support that approach and documents further a scrapie source which naturally affects GB ARQ/ARQ sheep.

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