Preparation of sections and immunohistochemical detection of abnormal prion protein

Instrumentation:

Five automated Ventana Discovery XT immunohistochemistry (IHC) instruments (Roche, Burgess Hill, UK) and identical protocols were used by the two screening laboratories. The entire immunolabelling process (dewaxing, antigen retrieval, antibody incubations, and haematoxylin counterstaining), was performed on Ventana Discovery XT automated biomarker platforms using Ventana Medical System reagents. Sections were dewaxed with EZ Prep Solution and the antigen retrieved by applying Cell Conditioning Solution Plus and Protease 3 (Ventana, UK). Nonspecific staining was reduced using Superblock (Medite, Switzerland).

Antibodies:

Abnormal prion protein (PrP) was detected using mouse monoclonal anti-PrP antibodies KG9 (PrP epitopes aa140-180; Dilution 1:500, TSE Resource centre, Roslin Institute Edinburgh, UK) on one section and ICSM35 (epitopes aa93-102; Dilution 1:1000 of 100µg/ml, D-Gen, UK) [10, 13, 14] on a second section and visualised using a peroxidase-diaminobenzidine (DAB) Detection Kit (DAB Map Ventana Medical System) (Fig 2A-D, F-I). To investigate suspect and confirm positive cases, the staining was repeated and additional anti-PrP monoclonal antibodies were applied (3F4, epitopes aa109-112; (Signet, UK) and 12F10 (epitopes aa142–160 Cayman Chemical, UK).

CD21 immunostaining was carried out with a prediluted Ventana staining cartridge according to the manufactures guidelines (Clone 2G9, order number 760-4245).

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PrP was detected using KG9 (1:1000 and later 1:500) and ICSM35 (1:1000 of 100µg/ml) and visualised using the DAB Map Detection Kit (Ventana Medical System). Sections were counterstained with haematoxylin and coverslipped (LEICA CV5030 coverslipper). Sections from sCJD brains (one with high and one with low PrP load) were used as a positive control for each machine cycle (30 slides); Omission of the primary antibody served as negative controls.

Additional Testing: Determination of PRNP codon 129 genotype

To determine the genotype of positive and suspect samples, allele discrimination using minor groove binding (MGB) probes with rtPCR was used as a primary assay for codon 129 analysis. This method has proven to be highly specific and sensitive in other studies. The ability of this technique to genotype small amounts of DNA extracted from formalin fixed, paraffin embedded appendices was assessed by firstly establishing whether complete concordance can be obtained between genotyped DNA extracted from fresh tissue and genotyped DNA extracted from the corresponding fixed tissue, and secondly the limit of the sensitivity of the technique to genotype accurately. The method was first validated using meningioma samples from the surgical pathology archive, and 100% concordance was established in 9 samples for which both fixed and fresh tissue was available. The limit of sensitivity was established by generating three standard curves, each representing one of the three genotypes with serial dilution from 20ng (estimated 6667 haploid genome copies) to an endpoint of 4.9pg (1.62 copies).The cycle threshold (C_t) values generated were used to define the C_t value that is likely to represent a single copy of target as well as establishing the point where allele drop out is a significant hindrance to accurate genotyping (i.e. a heterozygote would be miscalled as a homozygote). From the standard curves single copy amplification is centred around 40 cycles, the normal cycling limit for a real-time PCR assay. No blanks gave signals less than 40 cycles in any of the standard curves. The lowest acceptable C_t value for a sample was set at 37 cycles which represents about 6-7 copies. No allele drop out was recorded for the equivalent dilution point on the heterozygote standard curve and the C_t values for both alleles were above 37 suggesting a maximum C_t of 37 is an acceptable safety margin. There was no evidence of any allele signal in any well of any curve other than the ones from alleles known to be present i.e. there was no apparent stochastic contamination and there was always 100% allelic discrimination. Although homozygotes are more likely to genotype true they can be miscalled if there is stochastic contamination from the alternative allele. This will probably occur in only one of several replicates and is highly likely to be recognised as contamination because the C_t difference between alleles is likely to be a statistical outlier. As a further precaution, a rule was incorporated for any replicate to be ignored that has an aberrant C_t difference in a heterozygote call.

Adhering to the above criteria, DNA was extracted from one 10μm thick slice from each of the twenty fixed control appendices and assayed twice and the results compared. 1μl of 35μl of total DNA from each extraction was assayed in triplicate. From set 1, 15/20 were called and from set 2 14/20.Concordance was 100%.

The secondary assay used is a PCR based restriction endonuclease analysis, designed to incorporate the polymorphic Nsp1 site that defines the M129V polymorphism. The product was designed to incorporate a non-polymorphic Nsp1 site as an internal control. Both primers were labelled to increase the sensitivity of the assay by enabling use of the 3730xl DNA analyser. Standard curves similar to those run for the real-time assay were run to determine sensitivity. These suggest the assay will be 2-4 times less sensitive for heterozygote detection than the primary assay. Consequently only samples that were successfully genotyped on the real-time assay were carried forward. To avoid partial digests confounding genotyping, any replicate was ignored unless it conformed to a pre-set peak ratio. Rare stochastic contamination was filtered out in the same way. Adhering to the above criteria the 14 previously genotyped samples of set 2 were successfully re-genotyped independently by two researchers blinded to the genotypes of the primary assay. Concordance was 100%.

Supplementary Results:

Sample rejection and repeat staining (SM Table 2): For the samples examined at UCL-IoN, a detailed breakdown of the reasons for repeat stainings and causes of rejection was done. Of 20043 samples examined at UCL-IoN, 18% of the samples were rejected because of widespread necrosis due to an acute inflammation (i.e. appendicitis), which overran and destroyed the underlying normal follicular structures (Supplementary Fig. 2, Supplementary Table 1). 1.5% of the samples were rejected due to absence or too small an amount of lymphoid tissue without inflammation and 1.9% of the specimens did not contain appendix tissue, but represented other organs, probably due to incorrect coding of archived blocks or incorrect retrieval of blocks from the archives of participating hospitals. To obtain 40082 immunolabelled slides suitable for examination, 42037 sections had to be prepared, i.e. 1955 sections were repeats tests (repeat rate 4.7%; Supplementary Table 2). In addition, 3002 control sections were prepared. One negative and one positive control were present in each machine cycle (30slides). Of all repeats, 867 were for ICSM35 and 1088 for KG9 immunolabelling. The most common repeat reasons were machine cycle failure (54%), poor on-machine dewaxing (14%), DAB debris (12%), detached sections (5%) and miscellaneous (15%).

Supplementary Table 1: Previous UK Surveys of abnormal prion prevalence.										Comment
	Specimen	**Number collected**	**Number included in analysis**	**Positive**	**Technique**	**129 GT**	**Age group**	**Prevalence / Million population**	**95% CI (per million)**	
[1]	Appendix	4071	3075	0	IHC	n/a				Study reduced the upper bound on epidemic size is from several million cases to about 150 000 cases at the time (as of 1998)
[1]	Tonsil	95	95	0	IHC	n/a				
[2]	Appendix	14 964	12 674	3 /10 278	IHC	-/- VV VV	1961-1985 birth cohort	237	49–692	
				0 / 969	IHC		All samples			
	Tonsil	1739	1427	0 /694	IHC	n/a	1961-1985 birth cohort			
				0 /		n/a	All samples			
[3]	Tonsil	2000	2000	0	IHC WB	n/a	0-99 and above	0	n/a	

[4]	Tonsil	10 075	9672	1 (+2 ambiguous)	IHC	MV (MV) (VV)	1961-1985 birth cohort	109	3-608	
							All tested	103	3-576	
[5]	Tonsil	67 696	63007	0	EIA,		1961-1985 birth cohort	0	0-113	
Small case series or case reports:										
[6]	Tonsil	1	1	1/1	IHC, WB	129MM	1962			
[7]	Appendix	1	1	1/1	IHC		1953			
[8]	Tonsil	9	9	9/9	IHC (WB)	129MM	1963-1980			
	Spleen	10	10	10/10	IHC (WB)					
	Lymph node	8	8	8/8	IHC (WB)					
[1]	Appendix	1	1	0/1	IHC	n.d.				Case reported as part of a surveillance study. (Appendectomy 9 years prior to onset of vCJD,
[9]	Appendix	4	4	1 / 4	IHC, WB	129MM				Findings indicate that appendix does not reliably report vCJD infection even at the end stage of the disease.
[10]	**Ileum**	4	4	4/4	IHC, WB					

Supplementary Table 2: Analysis of inclusion and rejection rates for samples examined at UCL-IoN. Number of Appendix samples rejected, and stratification according to reason of rejection.

Appendix Tissue Rejection Rate	Number	%total
Total screened	20043	100%
Total suitable samples	15047	75.2%
Suitable samples that are not appendix tissue (i.e. Lymph nodes, tonsils)	126	0.6%
Rejected: Not appendix tissue:	438	2.2%
Total rejected	4969	24.8%
Analysis of inflammation in a subset of appendix samples		
Total samples assessed for inflammation	**14678**	**100%**
Suitable: Total	***11007***	75.0%
Suitable: non-inflamed (Supplementary Fig. 1A-C)	7375	67.0%
Suitable: mild to moderate inflammation (Supplementary Fig. 1D-F)	2239	20.3%
Suitable: severe inflammation (Supplementary Fig. 1G-I)	1393	12.7%
Rejected: Total	***3671***	***25.0%***
Rejected: No viable follicles, overrun and destroyed by inflammation	1880	12.2%
Rejected: Appendix with less than five follicles or atrophic tissue	1791	12.8%

Supplementary Table 3: Analysis of technical repeat reasons for samples examined at UCL-IoN. The total number of repeats was 1995 sections, and all % values are given as a fraction of this value

	ICSM35		**KG9**		**Combined**	
	Number	**%**	**Number**	**%**	**Number**	**%**

Total Repeats	867	44.3%	1088	55.7%	1955	100.0%
DAB debris	142	7.3%	94	4.8%	236	12.1%
Machine failures	543	27.8%	506	25.9%	1049	53.7%
Dewaxing	14	0.7%	250	12.8%	264	13.5%
Other (clearing, broken, levels)	31	1.6%	26	1.3%	57	2.9%
Poor reagent mixing	72	3.7%	146	7.5%	218	11.2%
Section detached	46	2.4%	47	2.4%	93	4.8%
QC sections	19	1.0%	19	1.0%	38	1.9%

Supplementary table 4: Participating hospitals

Hospitals	Post code	Trusts
Alexandra Hospital	B98 7UB	Worcestershire Acute Hospitals NHS Trust
City Hospital	NG5 1PB	Nottingham University Hospitals NHS Trust
Colchester General Hospital	CO4 5JL	Colchester Hospital University NHS Foundation Trust.
Cumberland Infirmary	CA2 7HY	North Cumbria University Hospitals NHS Trust
Darlington Memorial Hospital	DL3 6HX	County Durham and Darlington NHS Foundation Trust
Derriford Hospital	PL6 8DH	Plymouth Hospitals NHS Trust
Dewsbury and District Hospital	WF13 4HS	The Mid Yorkshire Hospitals NHS Trust
Epsom Hospital	KT18 7EG	Epsom and St Helier University Hospitals NHS Trust
Frenchay Hospital	BS16 1LE	North Bristol NHS Trust
Friarage Hospital	DL6 1JG	South Tees Hospitals NHS Foundation Trust
John Radcliffe Hospital	OX3 9DU	Oxford Radcliffe Hospitals NHS Trust
Maidstone Hospital	ME16 9QQ	Maidstone and Tunbridge Wells Hospitals NHS Trust
Manchester Royal Infirmary	M13 9WL	Central Manchester University Hospitals NHS Foundation Trust
Musgrove Park Hospital	TA1 5DA	Taunton and Somerset NHS Foundation Trust.
New Cross Hospital	WV10 0QP	The Royal Wolverhampton Hospitals NHS Foundation Trust
Pinderfields Hospital	WF1 4DG	The Mid Yorkshire Hospitals NHS Trust
Queen's Hospital	DE13 0RB	Burton Hospitals NHS Foundation Trust
Queen's Medical Centre	NG7 2UH	Nottingham University Hospitals NHS Trust
Royal Bolton Hospital	BL4 0JR	Bolton NHS Foundation Trust
Royal Cornwall Hospital	TR1 3LJ	Royal Cornwall Hospitals NHS Trust
Royal Derby Hospital	DE22 3NE	Derby Hospitals NHS Foundation Trust
Royal Devon & Exeter Hospital	EX2 5DW	Royal Devon and Exeter NHS Foundation Trust

Royal Lancaster Infirmary	LA1 4RP	University Hospitals of Morecambe Bay NHS Foundation Trust
Royal Shrewsbury Hospital	SY3 8XQ	The Shrewsbury and Telford Hospital NHS Trust
Royal Sussex County Hospital	BN2 5BE	Brighton and Sussex University Hospitals NHs Trust
Salisbury District Hospital	SP2 8BJ	Salisbury NHS Foundation Trust
Southampton General Hospital	SO16 6YD	University Hospital Southampton NHS Foundation Trust
St Thomas' Hospital	SE1 7EH	Guy's and St Thomas' NHS Foundation Trust
Stafford Hospital	ST16 3SA	Mid Staffordshire NHS Foundation Trust
Sunderland Royal Hospital	SR4 7TP	City Hospitals Sunderland NHS Foundation Trust
The James Cook University Hospital	TS4 3BW	South Tees Hospitals NHS Foundation Trust
Torbay Hospital	TQ2 7AA	South Devon Healthcare NHS Foundation Trust
Tunbridge Wells Hospital	TN2 4QJ	Maidstone and Tunbridge Wells Hospitals NHS Trust
University Hospital of North Durham	DH1 5TW	County Durham and Darlington NHS Foundation Trust
Whipps Cross Hospital	E11 1NR	Barts Health NHS Trust (*previous* Whipps Cross University Hospital NHS Trust)
Worcestershire Royal Hospital	WR5 1DD	Worcestershire Acute Hospitals NHS Trust
Wycombe Hospital	HP11 2TT	Buckinghamshire Healthcare NHS Trust
Yeovil District Hospital	BA21 4AT	Yeovil District Hospital NHS Foundation Trust

Supplementary figure legends

Supplementary Figure 1: Purulent inflammation of the appendix (i.e. appendicitis) overrunning and destroying secondary follicles. Follicular dendritic cells (FDC), detectable by immunohistochemical staining for CD21, disappear with progressive inflammation. A, B, C; No or minimal inflammation in an appendix biopsy: Secondary follicles are abundant and can be easily detected with a CD21 immunostain (B). C, high power magnification of one of the follicles shows a dense network of FDC, similar to the staining pattern of abnormal prion protein (see Fig 2). D, E, F: Moderate inflammation has destroyed the majority of secondary follicles and only occasional remnants of CD21 positive FDC are detectable F). Severe appendicitis with massive purulent inflammation with complete destruction of the follicular structures and abolished FDC, justifying the exclusion of inflamed appendix biopsies from the study. Red squares in B, E, H correspond to figures C, F, I. A, D, G; H&E staining, B, C, E, F, G, I; CD21 immunostaining. Scale bar: A, D, G; 300µm B, E, H; 3mm, C, F, I; 150µm.

J; Semi-quantitative determination of the inflammation in 7825 consecutive appendix biopsies during the study. The majority show no or minimal inflammation, and a smaller proportion shows purulent inflammation, in keeping with the clinical diagnosis of an appendicitis.

The rejection rate varied between different bins of 50 samples and also varied between different referring hospitals (Mean 0.19, Standard deviation 0.11; minimum 0 and maximum 0.58.The box plot indicates the values in the 2nd and 3rd quartile (>0.10 and <0.26, respectively).

Supplementary Figure 2: Non-specific labelling of structures within and outside of follicles in appendix samples with the antibody ICSM35. A-D and F-I, structures that are typically observed within follicles are macrophages (A, F), debris (B, G) and (myo) fibroblasts(C, H). D, I; non-specific deposition of DAB reagent in the centre of a follicle, lacking the typical FDC structure. Outside the follicles, fibroblasts and occasional nerve endings (E, J) were frequently observed. Scale bar: 400µm (A-E), 40µm (F-I) and 80µm (J).

Supplementary Figure 3: Comparison of the *PRNP* codon 129 genotype in the UK population with the frequency of the positive samples in this study and vCJD patients. Column 1 shows the frequency of the alleles on *PRNP* codon 129 UK population [19]. Column 2 shows the distribution of the genotypes in the positive samples of this study and column 3 the genotype of all 177 vCJD patients to date (i.e. all are *PRNP* codon 129MM).

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