

Prevalence of disease related prion protein in anonymous tonsil specimens in Britain: cross sectional opportunistic survey

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ABSTRACT

Objective To establish with improved accuracy the prevalence of disease related prion protein (PrP^{CJD}) in the population of Britain and thereby guide a proportionate public health response to limit the threat of healthcare associated transmission of variant Creutzfeldt-Jakob disease (vCJD).

Design Cross sectional opportunistic survey.

Study samples Anonymised tonsil pairs removed at elective tonsillectomy throughout England and Scotland.

Setting National anonymous tissue archive for England and Scotland.

Main outcome measure Presence of PrP^{CJD} determined by using two enzyme immunoassays based on different analytical principles, with further investigation by immunohistochemistry or immunoblotting of any samples reactive in either assay.

Results Testing of 63 007 samples was completed by the end of September 2008. Of these, 12 753 were from the birth cohort in which most vCJD cases have arisen (1961-85) and 19 908 were from the 1986-95 cohort that would have been also exposed to bovine spongiform encephalopathy through infected meat or meat products. None of the samples tested was unequivocally reactive in both enzyme immunoassays. Only two samples were reactive in one or other enzyme immunoassay and equivocal in the other, and nine samples were equivocally reactive in both enzyme immunoassays. Two hundred and seventy six samples were initially reactive in one or other enzyme immunoassay; the repeat reactivity rate was 15% or less, depending on the enzyme immunoassay and cut-off definition. None of the samples (including all the 276 initially reactive in enzyme immunoassay) that were investigated by immunohistochemistry or immunoblotting was positive for the presence of PrP^{CJD}.

Conclusions The observed prevalence of PrP^{CJD} in tonsils from the 1961-95 combined birth cohort was 0/32 661 with a 95% confidence interval of 0 to 113 per million. In the 1961-85 cohort, the prevalence of zero with a 95% confidence interval of 0 to 289 per million was lower than,

but still consistent with, a previous survey of appendix tissue that showed a prevalence of 292 per million with a 95% confidence interval of 60 to 853 per million. Continuing to archive and test tonsil specimens, especially in older birth cohorts, and other complementary large scale anonymous tissue surveys, particularly of post-mortem tissues, will further refine the calculated prevalence of PrP^{CJD}.

INTRODUCTION

Although the risk to the population of Britain of dietary exposure to the bovine spongiform encephalopathy agent that causes variant Creutzfeldt-Jakob disease (vCJD) has been virtually eliminated, the occurrence to date of four cases of vCJD infection resulting from blood transfusion has made real the threat of a secondary epidemic through healthcare associated human to human transmission.¹⁻⁴ These cases from blood transfusion have also established the existence of an infective asymptomatic stage in human vCJD. Estimating the prevalence of this asymptomatic infective stage, although technically challenging, is essential to guide a proportionate public health response to reduce the risk of healthcare associated transmission.

Measurement of prevalence in the 1961-85 birth cohort is a priority, given that 138 of the 167 cases of vCJD to date in Britain have been in this group (with 39 cases in the 1961-9 and 99 in the 1970-85 birth cohorts). Data are available from previous analyses of appendix and tonsil specimens for the presence of disease related prion protein (designated PrP^{CJD}) by immunohistochemistry and immunoblotting.^{5,6} The first study screened 11 247 appendix specimens and 1427 tonsil specimens by immunohistochemistry and found three positives in the appendixes from the 1961-85 birth cohort, giving a prevalence of 292 (95% confidence interval 60 to 853) per million.⁵ A second study found no positives in 2000 tonsil specimens screened by both immunohistochemistry and immunoblotting;⁶ half of these tonsils were from patients aged over

9 years and hence in the birth cohort likely to have had dietary exposure to bovine spongiform encephalopathy. Uncertainty about the true prevalence was increased when back calculation using plausible assumptions from the observed clinical vCJD cases suggested a much lower prevalence of sub-clinical vCJD infection than would be predicted from the finding of PrP^{CJD} in three appendixes.⁵⁷

The absence of a suitable blood test for PrP^{CJD}, and doubt about the clinical interpretation for a patient of a positive test result from testing any tissue, created major organisational and technical challenges for our large scale prevalence survey of PrP^{CJD}. To facilitate semi-automated enzyme immunoassay screening, we chose anonymised surgically removed tonsil pairs collected prospectively for the study reported here, rather than appendix tissue already archived in paraffin blocks that would have needed more labour intensive and slower immunohistochemical screening. PrP^{CJD} is known to accumulate to relatively high levels in the tonsils of people with vCJD, although, because of the difficulty of identifying such cases, it has not yet been shown to be present pre-clinically.^{8,9}

Commercially available enzyme immunoassay kits are routinely used for testing for bovine spongiform encephalopathy, scrapie, and other animal prion diseases; however, when our survey began no validated kits were available for testing human samples for PrP^{CJD}. We therefore issued a formal tender calling for manufacturers to take part in an enzyme immunoassay selection study and to supply suitable kits. The companies that responded were each sent two blinded panels of samples. Two assays, from Microsens and Bio-Rad, were able to detect brain from vCJD cases diluted 10⁻³ and spleen diluted 10⁻² into tonsil homogenate (Jillian Cooper, personal communication), and we selected these for use in this study. We now report the results of testing of the first 63 007 specimens from the intended collection of 100 000 in a national anonymous tissue archive.

METHODS

Test validation

We obtained unfixed palatine tonsil samples from 32 sheep with scrapie and 10 that were uninfected, as well as aliquots of unfixed frozen tonsil tissue taken at autopsy from six patients who died of vCJD. We prepared 12% homogenates from these and tested them by both enzyme immunoassays after making a dilution series from 10⁻¹ to 10⁻⁵ with negative human tonsil homogenate. We used a panel of 250 human tonsils that had been previously tested and found to be negative by immunoblotting and immunohistochemistry as examples of “true” negative controls.⁶

Survey tissue samples

Paired tonsil samples from people of all ages, and from operations done between January 2004 and September 2008, were collected from hospitals throughout England and Scotland. One tonsil of the pair was collected as fresh tissue chilled to 4°C, and the other tonsil was

collected in formalin. Tonsils arrived at the study centre an average of 65 (mode 50, median 113) hours after operation. Once transferred to suitable containers, samples were stored either at -80°C (fresh tissue) or at room temperature (fixed tissue).

Patients or their carers were given a leaflet explaining the aims of the study and that any result from testing their tonsil could not be traced back to them. An explicit paragraph and tick box to exercise a right to opt out of inclusion in the survey was included in the pre-tonsillectomy consent forms.

Investigatory algorithm

We homogenised a specimen of each tonsil pair and screened it with both enzyme immunoassays. We defined samples as “reactive,” “high negative,” or “negative” by a calculation based on the optical density readings from enzyme immunoassay for each microtitre plate. A reactive sample was within three standard deviations of the cut-off, and a high negative was within four standard deviations. We further investigated all samples that were initially reactive in either enzyme immunoassay or gave a high negative result in both enzyme immunoassays by immunoblotting and immunohistochemistry. We re-tested any sample that was high negative in one or other enzyme immunoassay by both enzyme immunoassays, and if it gave a reactive or high negative result in either we investigated it further by immunoblotting and immunohistochemistry. On occasion, we repeated immunoblotting tests with the same and with alternative antibodies.

Definition of a positive result

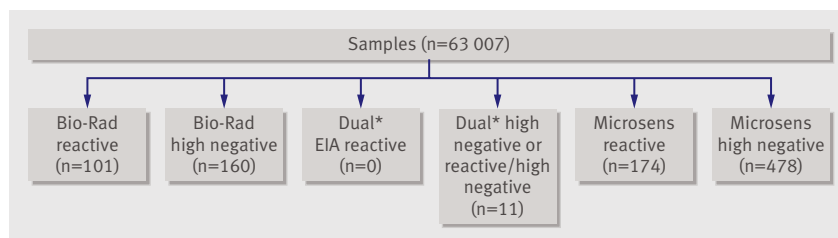
We defined a tonsil positive for PrP^{CJD} as one identified by enzyme immunoassay that was immunohistochemistry positive, had the expected specific protein band pattern in immunoblotting, or both.

RESULTS

Test performance

At a dilution of 10⁻³, 31 of 32 scrapie sheep samples were reactive in both enzyme immunoassays, and at a 10⁻⁴ dilution 21 were reactive in the Microsens enzyme immunoassay and 16 were reactive in the Bio-Rad enzyme immunoassay. One positive sample was detectable only at a dilution of 10⁻¹. Dilutions of 10⁻² and 10⁻³ could be detected by immunoblotting.

The six tonsil aliquots from human vCJD cases varied in the amount of lymphoid germinal centre tissue that was present, as judged by visual inspection. Depending on the quality of the tissue, PrP^{CJD} was detectable down to a dilution of 10⁻³ in the Microsens enzyme immunoassay and 10⁻² in the Bio-Rad enzyme immunoassay (table 1). The amount of PrP^{CJD} detected varied, as judged by the optical density values. This variation may have been due to biological differences in some cases, but an important contributory factor will have been the quality of the available tissue. Immunoblotting of aliquots of the vCJD samples showed that the expected specific band patterns of PrP^{CJD} were



Enzyme immunoassay screening of human tonsil tissue homogenates for PrP^{CJD}. *Dual enzyme immunoassay (EIA) reactive samples gave optical density readings above the cut-off classified as “reactive” in both Bio-Rad and Microsens tests; dual high negative or reactive/high negative samples gave optical density readings above the cut-off classified as “high negative” in both Bio-Rad and Microsens tests or was reactive in one and high negative in the other. All EIA reactive samples and most high negative samples were subject to both immunoblotting and immunohistochemistry testing (see text)

detectable. The sensitivities of the enzyme immunoassays were comparable to the immunoblotting results.

Survey specimens collected

Between January 2004 and October 2008, a total of 67 696 tonsil pairs had been archived after collection from 134 hospital trusts throughout England and Scotland. We received forms without tonsil tissue for 1426 patients who objected and 762 in whom clinical pathology examination had been requested. All regions of England contributed samples, and 5651 came from Scotland between January 2006 and September 2008.

We also tested another 2015 anonymous specimens, from tonsillectomies done in the southeast of England between July 2000 and August 2002, of which half were from patients aged over 9 years at operation, and that were untested as part of an earlier survey.⁶

Table 1 Enzyme immunoassay results on available tonsil tissue from six variant Creutzfeldt-Jakob disease (vCJD) cases (including sample of brain from one case)*: highest dilutions for reported result

Dilution†	Bio-Rad		Microsens	
	Optical density	Interpretation	Optical density	Interpretation
Specimen 1:				
Tonsil 10 ⁻²	0.06	High negative	0.12	Reactive
Brain 10 ⁻³	0.39	Reactive	0.08	Reactive
Specimen 2:				
Tonsil 10 ⁻²	0.04	Negative	0.11	Reactive
Specimen 3:				
Tonsil 10 ⁻²	0.06	High negative	0.20	Reactive
Specimen 4:				
Tonsil 10 ⁻¹	0.04	Negative	0.10	Reactive
Specimen 5:				
Tonsil 10 ⁻³	0.04	Negative	0.09	Reactive
Specimen 6:				
Tonsil 10 ⁻¹	0.13	Reactive	0.21	Reactive

*Three specimens supplied by National CJD Surveillance Unit (including paired tonsil and brain) and three by MRC Prion Unit.

†Dilution from 12% homogenate (10⁰); 10⁻¹ dilution is therefore equivalent to 0.012 g/ml vCJD tonsil tissue homogenate; as dilution is in negative homogenate, total tissue concentration was 0.12 g/ml for all samples tested.

Enzyme immunoassay screening results

By the end of September 2008, we had screened 63 007 samples with both enzyme immunoassays and, where indicated, completed investigatory testing (figure).

In one or other of the enzyme immunoassays, 276 samples gave an optical density defined as reactive and 638 were classed as high negative (figure). To define the repeat reactivity rate by enzyme immunoassay, we retested 487 reactive and high negative samples by enzyme immunoassay at the beginning of the project, before immunohistochemistry and immunoblotting confirmatory testing. The repeat reactivity rate was 15% (7/48) for the initially reactive samples and 3.5% (4/116) for the initially high negative samples in the Bio-Rad enzyme immunoassay. The equivalent figures for the Microsens enzyme immunoassay were 12% (7/60) and 10% (26/263). All initially reactive samples and any initially high negative samples that gave a repeat reactive or high negative result by enzyme immunoassay were subject to immunohistochemistry and immunoblotting confirmatory testing. Any samples that were initially reactive or high negative but which were not repeat tested by enzyme immunoassay went directly for immunohistochemistry and immunoblotting (figure).

No samples were clearly reactive in both enzyme immunoassays. One was reactive by Microsens and high negative by Bio-Rad, and another was reactive by Bio-Rad and high negative by Microsens. Nine were high negative by both the Microsens and Bio-Rad enzyme immunoassays. Seven of these 11 samples were methionine homozygote at codon 129 of the prion protein gene (*PRNP*) and four were heterozygote; only four (three homozygote and one heterozygote) were from people born before 1996 and therefore likely to have had dietary exposure to bovine spongiform encephalopathy.

Immunoblotting results

We demonstrated satisfactory immunoblotting performance, using two different protocols in two separate laboratories, by testing the tonsil tissue taken at autopsy from vCJD patients, as well as by spiking experiments using scrapie sheep tonsil tissue, scrapie infected hamster brain, and human vCJD brain tissue.

None of the survey sub-sample investigated by immunoblotting gave a protein banding pattern consistent with the presence of PrP^{CJD}. Some samples that showed a single band, which was not consistent with any expected pattern, were re-tested by immunoblotting either with the same antibody or with different antibodies, including 3F4 and a secondary antibody designed to reveal non-specific antibody interactions. Only one sample still showed a single immunoblotting band; it was methionine homozygote at codon 129 and from a patient in the 1986-90 birth cohort, and it was negative by immunohistochemistry.

Immunohistochemistry results

More than 800 tonsils, selected on the basis of the enzyme immunoassay results, have been investigated

by immunohistochemistry in one or other of two experienced laboratories, and none was scored positive for PrP^{CJD}.

Prevalence estimates

Overall, 32 661 (52%) of the 63 007 samples tested came from people born in 1995 or earlier who were alive at the time when bovine spongiform encephalopathy contaminated meat was being consumed (table 2). The observed prevalence of PrP^{CJD} in this group was zero (95% confidence interval 0 to 113 per million). Combining the 1986-90 and 1991-5 cohorts gave a prevalence of zero with an upper 95% confidence limit of 185 per million. The prevalence in the combined 1996-2000 and 2001-7 unexposed cohorts was also zero with an upper 95% confidence limit of 122 per million.

Although the zero per million prevalence seen in the 1961-85 cohort (upper 95% confidence limit 289 per million) was different from the 292 per million (95% confidence interval 60 to 853 per million) found in the earlier survey of appendix tissue,⁵ the 95% confidence intervals for both surveys overlapped (a formal comparison of the prevalence estimates gives a P value of 0.09).

DISCUSSION

Initial results from testing the tonsil specimens in a national anonymous tissue archive have shown the prevalence of PrP^{CJD} to be zero in 63 007 overall and zero in 12 753 in the birth cohort in Britain in which most cases of vCJD have occurred. Interpretation of this finding, and of the difference between it and the earlier survey of appendix tissue, depends critically on three factors: the sensitivity of the test system chosen to screen the tonsil specimens, the representativeness of the sample specimens of the people most vulnerable to vCJD disease, and the natural history of the infectivity of bovine spongiform encephalopathy in individual patients, particularly the time when PrP^{CJD} first appears pre-clinically in tonsil compared with appendix tissue and how long it persists.

Test sensitivity

Three experiments investigated the sensitivity of the enzyme immunoassays. The first was the enzyme immunoassay selection study, the second was the interrogation of the enzyme immunoassays with tonsil tissue from sheep with scrapie, and the third was the use of tonsil tissue from patients who died from vCJD. Overall, these indicated that the Microsens enzyme immunoassay was more sensitive than the Bio-Rad enzyme immunoassay for detection of PrP^{CJD} in lymphatic tissue. The most sensitive detection was by the Microsens enzyme immunoassay with a sample containing 12 µg vCJD tonsil tissue; the equivalent for the Bio-Rad enzyme immunoassay was 480 µg vCJD tonsil tissue (table 1). When used for screening, 12 000 µg tonsil tissue was applied to the Microsens enzyme immunoassay and 48 000 µg to the Bio-Rad enzyme immunoassay. Therefore, the two enzyme immunoassays should have been sufficiently sensitive to detect PrP^{CJD} in tonsils from asymptomatic people incubating vCJD if levels of PrP^{CJD} were a 10th to a 1000th of those in patients with symptoms.

The dual enzyme immunoassay tonsil screening protocol may be at least as sensitive as any other large scale testing for abnormal prion protein that could have been used. The enzyme immunoassays use different test principles and antibodies, perhaps reinforcing the sensitivity of each. Reading of the results was automated, and we used a range of controls on each 96 well plate of tests. We deemed the use of a single enzyme immunoassay cut-off value as commonly applied to screen a population with many positives to be inappropriate, as this particular set of samples was expected (and found) to be overwhelmingly negative. Therefore, we calculated the cut-off value for each plate individually, and this method almost doubled the number of specimens that were selected for further investigation by immunoblotting and immunohistochemistry.

Several reasons exist why a specimen could have given a false high (reactive or high negative) optical density reading in either or both enzyme immunoassays: inadequate proteinase K digestion of PrP^C (the normal cellular form of PrP) for the Bio-Rad enzyme immunoassay, inadequate removal of PrP^C bound to the capture polyanion for the Microsens enzyme immunoassay, non-specific antibody interactions owing to the high antibody concentration in tonsil tissue, and poor sample quality or technical failures. Therefore, applying more specific immunoblotting and immunohistochemistry tests to confirm whether PrP^{CJD} was present was essential.

In comparison with immunohistochemistry, the volume of tonsil tissue screened by enzyme immunoassay was relatively large. Immunohistochemistry on appendix tissue may also be less specific than immunoblotting, so that prevalence estimated by immunohistochemistry screening may tend to overestimate the true situation.⁹ However, to tackle the lingering uncertainty that screening immunohistochemistry might be more sensitive than dual enzyme immunoassay

Table 2 | Prevalence of disease related prion protein (PrP^{CJD}) in Britain by birth cohort (positive/total; rate per million with 95% confidence intervals*)

Birth cohort	Current (2004-September 2008) national tissue survey: tonsils	Earlier† (1995-9) national tissue survey	
		Appendices	Tonsils
1940 and before	NA	NA	0/225
1941-60	NA	0/573	0/266
1961-85	0/12 753; 0 (0 to 289)	3/10 278; 292 (60 to 853)	
1986-90	0/9 564; 0 (0 to 386)	0/396	0/119
1991-5	0/10 344; 0 (0 to 357)	NA	0/106
1996-2000	0/15 708; 0 (0 to 253)	NA	0/17
2001-7	0/14 638; 0 (0 to 252)	NA	NA
Total	0/63 007; 0 (0 to 59)	3/11 247; 267 (55 to 779)	0/1 427; 0 (0 to 2 582)

NA=not available.

*95% confidence interval calculated only when denominator exceeds 1000.

†Data from separate tissue survey of 2000 tonsils (July 2000-August 2002) in southeast England (including London)⁶ not included.

screening, a further study to re-test 10 000 of the archived tonsils by immunohistochemistry has been commissioned. These 10 000 samples comprise those from patients in the 1961-85 birth cohort, as well as any samples that gave optical density readings above the cut-offs in either of the two enzyme immunoassays. The results from this major undertaking should be available some time during 2009.

Two of the three positive samples in the retrospective immunohistochemistry study of appendix tissue were valine homozygous at codon 129 of *PRNP*.^{5,10} Therefore, we can be confident that the antibodies used in our immunohistochemistry analysis would have showed PrP^{CJD} in a valine homozygote if it was present. The antibodies used in the enzyme immunoassay and immunoblotting would similarly be likely to detect PrP^{CJD} in a valine homozygote and, by extension, PrP^{CJD} in a heterozygote. Although the immunoblotting profiles of valine homozygote and heterozygote vCJD are unknown, they may be expected to consist of three or four glycoforms.¹¹ The immunoblotting profile of the spleen in a case of asymptomatic vCJD infection in a heterozygote patient showed similarities to that in clinical vCJD spleen samples in methionine homozygote patients, with a predominance of the diglycosylated band.² We did not observe by immunoblotting any pattern similar to any recognised profiles in sporadic CJD or vCJD.¹²⁻¹⁶ The only repeatedly anomalous immunoblotting pattern seen was of a single immunoblotting band in an immunohistochemistry negative sample, which was methionine homozygote at codon 129 of *PRNP*.

Representativeness of sample

The age and sex characteristics of the samples in our study reflected the current age and sex distribution of people having tonsillectomy: 72% of those born in 1995 or earlier in our survey were female, compared with 48% of those born since 1995. Although only 44% of vCJD cases to date have been in women, we do not think that the predominance of females in our older sample of tonsils could have biased our findings with respect to prevalence of PrP^{CJD}.

Given the very strong association between PrP^{CJD} and people who are homozygous for methionine at *PRNP* codon 129,⁵ it is important to note that our sample was likely to have been representative of this genetic susceptibility: an analysis of 466 of the tonsils in our survey showed 47% to be methionine homozygotes at codon 129, consistent with what was expected.^{10,17-20} Therefore, of the 32 661 tonsils tested from people born before 1996, approximately 15 351 (47%) would have been from methionine homozygotes.

Several differences must be considered when comparing results between surveys. First and foremost is that previously appendix tissues were screened by immunohistochemistry, whereas we screened tonsil tissue by enzyme immunoassay. Secondly, an average of 10 years elapsed between when the previous large

sample from the 1961-85 birth cohort had their appendixes removed (during 1995-9) until our sample had their tonsils removed (mostly in 2006-7)—10 years during which abnormal prion protein levels might be expected to have increased rather than diminished. Within this birth cohort, however, the average age of appendectomy was estimated to be four years older than the average age of tonsillectomy, so the average duration of the opportunity for PrP^{CJD} to increase between the appendectomy samples and the tonsillectomy samples would have been about six years. On the other hand, the relatively older appendix sample that was collected earlier may conceivably have contained a wave of infectivity in the 1961-85 cohort of the British population that was not present in the younger tonsil group that was sampled later.

Detailed information on previous operative history was sought on every vCJD case diagnosed in Britain. Seventeen of 167 patients were reported to have had tonsillectomy; 14 of these were in the 1961-85 birth cohort, and the remaining three were in the pre-1960 birth cohort. None was likely to have had specimens included in this or the earlier tonsil survey (Hester Ward, personal communication).⁶

Natural history

While PrP^{CJD} has been found consistently by immunoblotting and immunohistochemistry in tonsil tissue from patients with vCJD,^{8,9,21-24} PrP^{CJD} in a tonsil from an asymptomatic person has yet to be reported. Given, however, that tonsillar tissue has been shown to accumulate PrP^{Sc} before the onset of clinical disease in non-human primates and well before the onset of clinical disease in sheep experimentally infected orally with bovine spongiform encephalopathy,^{25,26} we considered tonsil tissue to be a reliable substrate for a survey of prevalence in humans. Also, the use of fresh tonsil tissue allowed more comprehensive laboratory testing, if necessary, after the initial screening assays.

PrP^{CJD} has been observed to accumulate in appendix tissue in vCJD (19/20 positive/tested)^{9,27,28} and, in two cases, before symptoms developed.^{29,30} However, data on the timing of the appearance of PrP^{CJD} in different peripheral lymphoreticular tissues during the prolonged incubation period of vCJD are sparse. The rate of accumulation of PrP^{CJD} in tonsil and appendix tissue could differ such that the findings of surveys of appendix and tonsil tissues would also differ. The positive samples found in the appendix survey presumably came from people who were infected a relatively short time earlier, during the peak of the bovine spongiform encephalopathy epidemic.⁵ Moreover, should the incubation period for prion disease be considerably longer in people with different genotypes, uncertainty about the timing of the appearance of detectable PrP^{CJD} in these will increase, with concomitant implications for the interpretation of results of PrP^{CJD} prevalence surveys.

Animal experiments have shown that high infectivity, and indeed disease, can be present in the absence of detectable proteinase K resistant PrP^{Sc}.³¹ The extent to

WHAT IS ALREADY KNOWN ON THIS TOPIC

Statistical back calculation based on cases of vCJD to 2004 has given estimates of between 10 and 190 further clinical cases over the next few decades

A study of archived appendix and tonsil tissues found a prevalence of lymphoreticular accumulation of pathogenic prion protein consistent with the existence of between 520 and 13 000 sub-clinical cases

Therefore, a discrepancy exists between estimates, which needs to be resolved to ensure that proportionate public health measures are implemented

WHAT THIS STUDY ADDS

Testing of tissue from more than 63 000 tonsils, of which 12 763 were from the 1961-85 birth cohort, has not shown evidence for the presence of the pathogenic form of the prion protein

The prevalence of sub-clinical vCJD infection in Britain may be lower than that given by previous estimates, with an upper limit of 289 per million in the 1961-85 birth cohort

which this observation can be generalised is, however, unclear, as PrP^{CJD} has been shown to be present in the lymphoid tissues of all vCJD patients tested.^{9,27} If other, more reliable, indicators of vCJD become available, screening the existing samples with tests for these markers, and thereby determining whether any vCJD positives have been missed by looking only for PrP^{CJD}, may be possible.

Data from animal experiments also show “clearance” of abnormal prion protein after inoculation.^{31,32} Therefore, the abnormal prion protein found in the earlier survey of appendix tissue may conceivably have been transient and eventually cleared without leading to disease, so that the appendix survey result would not have been replicated by the later tonsil survey.

Conclusion

We tested more than 32 000 tonsils from people in the age range most exposed to meat contaminated with bovine spongiform encephalopathy, and believed to be asymptomatic when sampled, for disease related prion protein. Using two sensitive enzyme immunoassays, with selective application of specific immunoblotting and immunohistochemistry techniques, we found no samples positive for PrP^{CJD}, a prevalence of 0 per million (with an upper 95% confidence limit of 113 per million). For the 1961-85 birth cohort, the prevalence of zero with a 95% confidence interval of 0 to 289 per million was lower than, but still consistent with, the earlier study of appendix tissue (60 to 853 per million). A P value of 0.09 applies to the comparison of the two prevalence estimates. These two surveys may not, however, be directly comparable owing to differences in testing methods, tissues sampled, and the time the tissues were removed (typically about 10 years earlier in the previous study). More data are needed through continuing the testing of tonsils from people born before 1996, despite the low frequency of tonsillectomy in older birth cohorts. In addition, creation and testing of other anonymous tissue archives, such as one based on coronial autopsies, or a repeat of the appendix survey on an even larger scale, should provide a

larger sample set of the people most exposed to the bovine spongiform encephalopathy agent.³³

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Contributors: JPC designed and analysed the laboratory studies and wrote the paper with ONG, who initiated the study and did clinical and epidemiological analyses. CMK recruited hospitals to the study and did epidemiological analyses. NA did statistical and epidemiological analyses. KV organised the National Anonymous Tissue Archive laboratory, tonsil processing, and enzyme immunoassay testing. GM, MK, and RD did the immunoblotting. DAH, PE, JWI, LMCC, and DLR did the immunohistochemistry. JWI provided some of the vCJD clinical tissue used in the work. HEA did the codon 129 genotyping. JPC and ONG are the guarantors.

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Competing interests: None declared.

Ethical approval: The study received ethical approval from the Trent Multi-centre Research Ethics Committee (MREC/03/4/073). None of the participants in the study was subsequently identifiable.

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