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Original Paper

Prevalence of lymphoreticular prion protein accumulation in UK tissue samples

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Abstract

This study aims to provide an estimate of the number of individuals in the UK who may be incubating variant Creutzfeldt-Jakob disease and at risk of causing iatrogenic spread of the disease. Lymphoreticular accumulation of prion protein is a consistent feature of variant Creutzfeldt-Jakob at autopsy and has also been demonstrated in the pre-clinical phase. Immunohistochemical accumulation of prion protein in the lymphoreticular system remains the only technique that has been shown to predict neurological disease reliably in animal prion disorders. In this study, immunohistochemistry was used to demonstrate the presence of prion protein, with monoclonal antibodies KG9 and 3F4, in surgically removed tonsillectomy and appendicectomy specimens. The samples were collected from histopathology departments across the UK and anonymised prior to testing. Samples were tested from 16 703 patients (14 964 appendectomies, 1739 tonsillectomies), approximately 60% of whom were from the age group 20–29 years at operation. Twenty-five per cent of the samples were excluded from the final analyses because they contained inadequate amounts of lymphoid tissue. Three appendicectomy samples showed lymphoreticular accumulation of prion protein, giving an estimated prevalence of 3/12 674 or 237 per million (95% CI 49–692 per million). The pattern of lymphoreticular accumulation in two of these samples was dissimilar from that seen in known cases of variant Creutzfeldt-Jakob disease. Although it is uncertain whether immunohistochemical accumulation of prion protein in the lymphoreticular system is specific for variant Creutzfeldt-Jakob disease, it has not been described in any other disease, including other forms of human prion disease or a range of inflammatory and infective conditions. These findings reinforce the importance of measures taken by the UK Department of Health to reduce the risk of spread of variant Creutzfeldt-Jakob via blood products and surgical instruments, and of the urgency to proceed with large-scale screening of fresh tonsil specimens for the presence of prion protein.

Keywords: **Creutzfeldt-Jakob disease (CJD); prion; screening; immunohistochemistry**

Introduction

Variant Creutzfeldt-Jakob disease (vCJD) was first recognized as a new and distinctive disease in the UK in 1996 [1]. Subsequent transmission studies and strain typing have shown that the transmissible agent causing vCJD exhibits identical characteristics to the bovine spongiform encephalopathy (BSE) agent [2–4] and there is no evidence that vCJD occurred prior to 1995 [5,6]. These data indicate that vCJD is a new disease, almost certainly caused by exposure to the BSE agent. This conclusion has led to concern about a possible human epidemic of vCJD, particularly as it is likely that over 400 000 infected cattle entered the human food chain in the UK prior to the introduction of the specified bovine offal ban in November 1989 and as the ban was not fully effective for several years, a large number of infected cattle also entered the food chain in the early 1990s [7]. There have been a number of attempts to predict future numbers of vCJD cases using mathematical models and extrapolating from vCJD cases seen to date [8–13]. Recent estimates based on the pattern of clinical cases suggest that the epidemic of vCJD will be relatively small, with an upper 95% confidence interval of 540 future cases [13]. However, remaining uncertainties, including the possibility that other genetic loci affect susceptibility [14], make the distribution and timing of any human epidemic unclear. Furthermore, such models are unable to estimate the prevalence of asymptomatic infection and hence provide any estimate of the potential number of future infections and cases that could arise from secondary (human-to-human) transmission of vCJD. In addition, questions have been raised as to the

safety of some food products not covered by the specified bovine offal ban [15,16] and it is not known if BSE has entered the British sheep flock, factors which could alter predicted numbers of vCJD cases [12]. These uncertainties make decisions about health care planning problematic, particularly measures to reduce the risk of iatrogenic spread of vCJD. In order to reduce these uncertainties, some form of population screening is required. However, the lack of a conventional immune response and the failure to date to demonstrate abnormal prion protein (PrP) in blood in vCJD [17] have made the development of a diagnostic blood test difficult. If a blood test becomes available for symptomatic vCJD, it may be several years before it is known whether pre-clinical disease could be reliably detected.

It has been known for some time that lymphoreticular accumulation of PrP occurs early in murine models of scrapie [18], even when incubation periods are long [19]. This lymphoreticular involvement has been successfully used in the development of a tonsillar biopsy as a pre-clinical test for scrapie in sheep [20]. Although widespread lymphoreticular involvement is not a feature of BSE in cattle [21], extensive lymphoreticular PrP deposition has been found in all cases of symptomatic vCJD examined to date [22,23] and in two cases in appendicectomy specimens removed prior to the onset of symptoms [24,25]. On the basis of these data, we have screened large numbers of appendicectomy and tonsillectomy specimens for the presence of abnormal lymphoreticular PrP deposition. Although the antibodies used in this study cannot distinguish PrP^{c} from PrP^{Sc}, immunohistochemical accumulation of PrP within lymphoid tissue correlates with the detection of protease-resistant PrP by western blot analyses in human tissues [22] and immunohistochemistry remains the only technique that has been shown to predict disease in animals reliably [26,27]. This study was primarily designed to look for evidence of a large epidemic, but also to provide information about how many individuals are at high risk of developing vCJD and causing iatrogenic spread. Interim results from this study have been published previously [25,28]. However, the study has now been completed following the examination of additional cases.

Materials and methods

Tissue samples

Appendicectomy and tonsillectomy samples were identified by Systematized Nomenclature of Medicine (SNOMED) searching of the computerized databases of 63 histopathology departments across the UK. Initially, samples from the age range 10–50 years were included. However, following negative findings in the first 3000 cases [28], it was decided only to examine appendix samples from individuals aged 20–29 years, as this represents the highest risk age group for vCJD. Tonsil samples included all ages, as fewer samples were available for examination (most tonsillectomy samples are discarded rather than sent to histopathology departments for diagnosis and archiving). A maximum of two tissue blocks was examined for each case. Only samples removed from 1995 onwards were included, as these represent a longer time from possible BSE exposure than earlier samples and therefore a greater likelihood of PrP being detectable. Tissue samples were collected into batches of at least 1000 cases and given a randomly obtained study number prior to testing, in order to protect the anonymity of positive individuals. Batches of samples from England were tested at Plymouth and, from Scotland, at Edinburgh.

The study received approval from the South and West Multi-centre Research Ethics Committee (MREC reference 99/6/32) and for each of the centres included, appropriate local research ethics committee approval. The ethical approach has been discussed previously [29] and in view of the lack of direct patient consent and uncertainty of the significance of a positive result, the study design was anonymous.

Immunohistochemistry

Four-micrometre sections were cut from tissue blocks at two levels 100 μm apart. Sections were pretreated by autoclaving at 121 °C for 10 min, followed by immersion in 96% formic acid for 5 min and digestion with proteinase K (10 μg/ml) for 5 min at room temperature, in order to enhance PrP^{Sc} detection and reduce PrP^{c} detection. PrP was detected using the well-characterized and widely used monoclonal antibodies 3F4 (Dako, UK) and KG9 (IAH, TSE Resource Centre, UK) [22,24] and visualized using the CSA kit (Dako, UK), which gives superior results in terms of sensitivity to most other immunohistochemical detection systems [30]. A section from each case was stained with haematoxylin and eosin for morphological assessment. Autopsy tonsil tissues from confirmed cases of vCJD were used as a positive control for each group of slides stained by immunohistochemistry for PrP; negative controls were performed by omitting the primary antiserum. Thirty cases from each batch of 1000 were exchanged between the study centres and tested 'blinded' to the findings of the other centre, for quality control and validation of results. In order to minimize the possibility of human error, the samples were tested and analysed with each of the antibodies on separate dates.

All sections were examined by an experienced neuropathologist (DAH at Plymouth and JWI at Edinburgh). Cases with fewer than five secondary lymphoid follicles were excluded from the final analyses because in the original reported case [24] and those examined at autopsy (personal observation JWI), PrP could be demonstrated in only approximately 20% of follicles. Sections were recorded as positive if PrP staining was detected in follicular dendritic cells or tingible body macrophages in lymphoid follicles.

Statistical methods

Simple summary statistics were calculated in Microsoft Excel. Exact binomial confidence intervals were calculated for the prevalence estimates. The expected number of individuals incubating vCJD was calculated using estimates of the UK population size stratified by age (http://www.statistics.gov.uk/downloads/theme_population/PT114.pdf).

Results

Tissue samples

The numbers of cases examined and the age distribution are summarized in Table 1 and Figure 1. The age distribution of our sample is heavily weighted towards the high-risk age group (based on cases of vCJD to date, see Figure 2). The majority of the specimens examined were appendicectomies, reflecting the availability of samples within histopathology departments (most tonsillectomy specimens are discarded after surgery in the UK).

The number of secondary lymphoid follicles varied considerably between appendicectomy cases, but in about 25%, fewer than five were present on the first level and these were therefore excluded from the figures for analyses. Most of these excluded cases were severely inflamed, although some showed fibrous obliteration, and none was considered positive. The median number of secondary lymphoid follicles in the remaining appendicectomy cases, which were included in the study, was 22 at the first level and most had several additional follicles examined at the second level. Most tonsil specimens included over 100 secondary lymphoid follicles, although in about 10% of samples, fewer than five were present.

Table 1. Summary of the samples used in the study

Specimens	Number
Appendicectomy specimens tested	14 964
Tonsillectomy specimens tested	1739
Excluded from analysis*	4029
Total included in analysis	12 674†

* Due to inadequate amounts of lymphoid tissue.
† 10 260 from England and 2414 from Scotland.

Figure 1. Age distribution of the samples included in the study

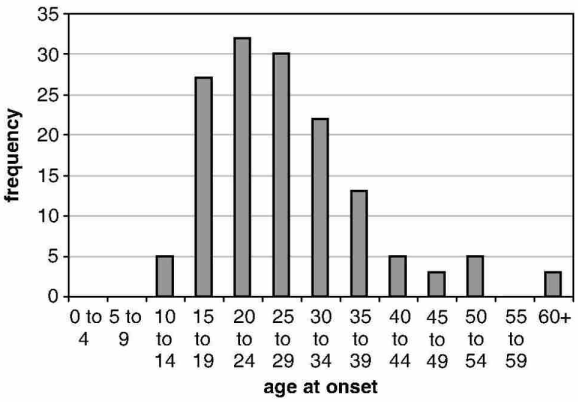


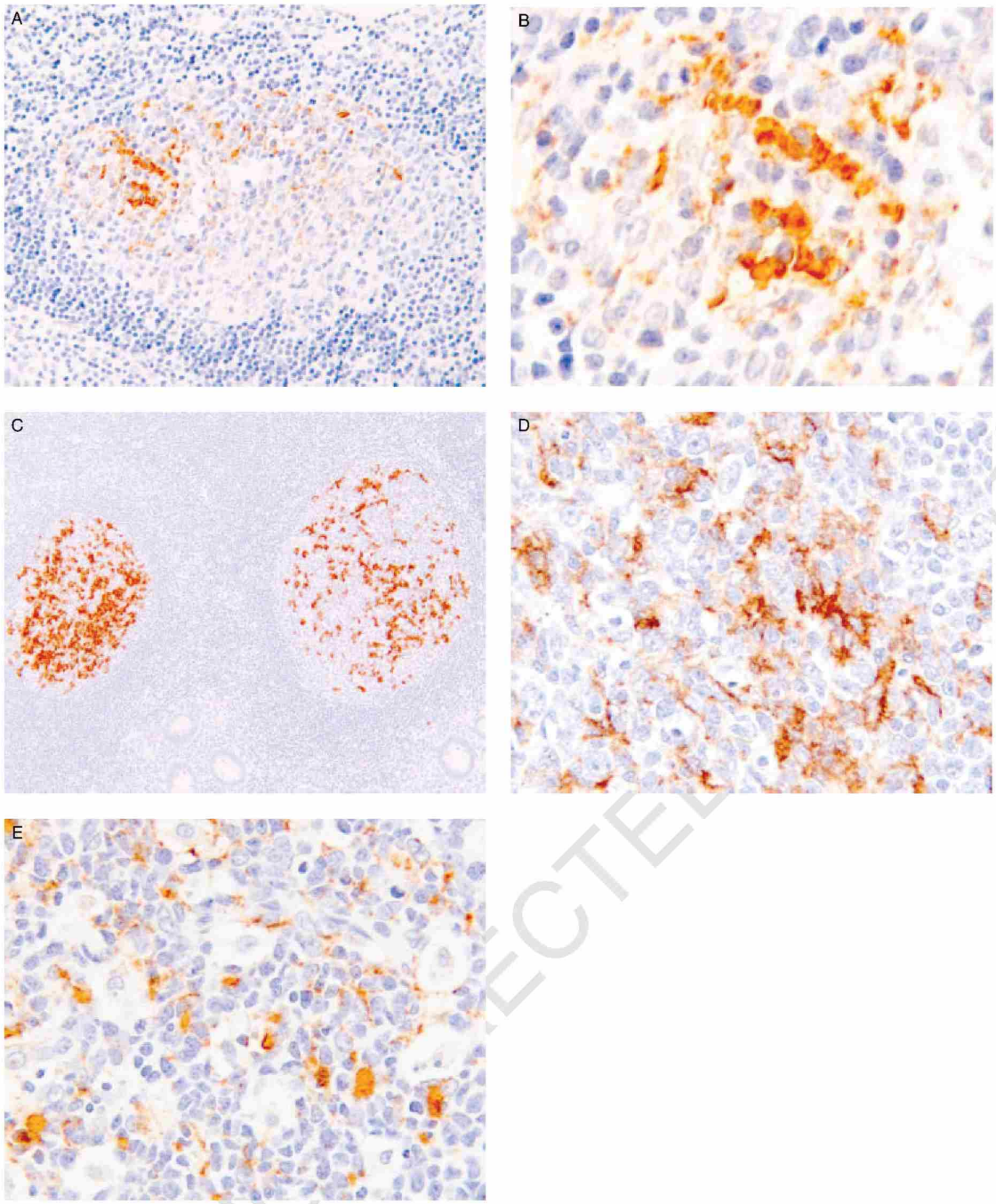
Figure 2. Age distribution at onset of vCJD cases to end of 2003

Immunohistochemistry

In the majority of samples, fine granular PrP immunoreactivity was noted in nerve fibres and the myenteric ganglia with both antibodies, and in a few cases, PrP immunoreactivity was also noted in epithelial cells immediately adjacent to acute inflammation. In three appendicectomy cases, we identified PrP immunoreactivity in lymphoid follicles, which was seen in sections tested at both centres. None of the tonsillectomy samples was positive.

In the first positive case (previously published [25]), immunoreactivity was seen in the sections stained using KG9 and was limited to one of the six secondary lymphoid follicles present, with a distribution suggesting that it was within follicular dendritic cells (Figure 3A). The pattern of staining, in particular the coarse granularity (Figure 3B), was very similar to that seen in the two other cases who subsequently developed vCJD [24,25]. However, staining was less evident in sections immunostained with the 3F4 antibody. The reason for this discrepancy is not entirely clear, although we feel that the most likely explanation is sampling error due to the focal nature of the PrP deposition. This positive case also showed evidence of acute appendicitis in adjacent tissue, but there was no morphological evidence of any other disease process in an adjacent haematoxylin and eosin-stained section.

The second positive sample showed extensive staining in 31 of 68 secondary lymphoid follicles (Figure 3C); this was seen with both antibodies, although it was less intense with 3F4. The staining had a finer granular pattern and appeared confined to follicular dendritic cells (Figure 3D). The appendix did not show any acute inflammation. A very occasional



Color Figure - Print and Online

Figure 3. Immunoreactivity with monoclonal anti-PrP antibody KG9 in the three study cases. The first positive case shows granular staining of follicular dendritic cells in one follicle (A), including numerous coarse granular aggregates (B). The second positive case shows intense PrP immunoreactivity in two follicles (C), with a predominantly finely granular pattern in follicular dendritic cells (D). The third case shows a mixture of granular follicular dendritic cell staining and accumulation within the cytoplasm of macrophages (E)

multinucleate cell was noted in the submucosa of this case, but not within germinal centres.

The remaining positive case showed staining in three of 46 secondary lymphoid follicles, which was similar with both antibodies. Fine granular immunoreactivity was present in cells with the morphology of follicular dendritic cells, and within the cytoplasm of cells with abundant eosinophilic cytoplasm, presumably macrophages (Figure 3E). Acute inflammation was not present.

If lymphoreticular immunoreactivity for PrP is a reliable marker of pre-clinical infection, the estimated prevalence of vCJD based on these three positive samples in 12 674 tested will be 237 infections per

million population (exact 95% CI 49–692 per million). If we assume that this estimate relates to those aged 10–30 years (83% of the sample), then this translates to a best estimate of 3808 individuals (95% CI 785–11 128) aged 10–30 years incubating vCJD. If only the one case with a similar pattern to that seen in previous cases of vCJD is considered, then the estimates will be correspondingly lower (prevalence of 79 infections per million population, 95% CI 2–440). In contrast to these high estimates, clinical case numbers remain at a much lower level and have been declining since 2000, with only 18 deaths in 2003.

Discussion

This study provides an estimate of the prevalence in the UK population of abnormal lymphoreticular accumulation of PrP. However, our findings need to be interpreted cautiously, in terms of the clinical significance of both negative and positive results.

One major limitation of this study in estimating the prevalence of asymptomatic infection and predicting future numbers of vCJD cases is that it is not known at what stage during the incubation period PrP can be detected in lymphoid tissue. In murine models of scrapie, infectivity can be demonstrated in Peyer's patches as early as 1 week after oral inoculation [19] and immunohistochemistry can detect PrP in Peyer's patches 1 month after intraperitoneal inoculation [31]. In the tonsils of scrapie-infected sheep, immunohistochemical detection of PrP occurs from 4 months of age in those homozygous for a susceptibility PrP gene polymorphism, and by 15 months in heterozygotes, reliably predicting future neurological disease [26]. A further study examining tissue from the third eye of sheep at risk of scrapie found that immunohistochemical detection of PrP in lymphoid follicles predicts neurological disease with an estimated 87% sensitivity and 94% specificity [27]. Data are only available in the pre-clinical phase from three cases of vCJD [25]; the two appendicectomy samples removed in the 1990s (up to 2 years before symptoms and 4 years before death) were positive and a third case, removed in 1987, 10 years before the onset of symptoms, was negative. This retrospective study has only examined samples taken from 1995 to 1999, several years after the peak human exposure to BSE, which is likely to have occurred between 1988 and 1992, in order to maximize the chances of identifying positive individuals. Furthermore, we have used a highly sensitive immunohistochemical technique [30] and because of the focal nature of PrP deposition, extensive sampling of appendix tissue, with a minimum of five (and an average of more than 20) secondary lymphoid follicles assessed in each case. Using this approach, we have found that 95% of autopsy appendicectomy samples from cases of vCJD, with adequate amounts of lymphoid tissue, test positive [25]. The finding of fine granular PrP in the myenteric plexus of most samples (and some epithelial cells adjacent to inflammation in a few samples) suggests that the proteolytic digestion used during immunocytochemistry does not completely remove PrP^c and also reflects the high levels of PrP^c in autonomic nerves [32].

Although immunohistochemical accumulation of PrP in lymphoreticular tissues has not been demonstrated in any disease other than vCJD [22,33,34], the significance of the positive samples in this study is not certain. In one case, the immunohistochemical pattern of immunoreactivity resembled that seen in appendix tissue from pre-clinical [24,25] and autopsied cases of vCJD, but in the other two cases, a more finely granular pattern of staining was present in relation to follicular dendritic cells, raising the possibility that these may be false positives. However, we have been unable to demonstrate PrP immunoreactivity in a range of other disorders including other human prion diseases, neoplastic disease, or a range of inflammatory conditions [33]. Other explanations for our finding of cases with an unusual pattern of lymphoreticular PrP immunoreactivity include involvement of other genotypes (genotype is known to affect the morphological patterns of PrP deposition in the brain [35]) or differing strains of BSE [36]. The anonymous study design prevents detailed investigation of the positive cases. However, spare paraffin wax sections were available from the second and third positive cases and have been used for transmission studies, but these may be inconclusive if negative, because of the small amount of tissue available and the difficulty in transmitting from fixed tissue [37]. Commercially available anti-PrP antibodies for immunohistochemistry detect both PrP^c and PrP^{Sc}, and although two groups have developed PrP^{Sc}-specific antibodies [38,39], they do not appear to work for immunohistochemistry (JWI, personal communication).

If our positive cases represent pre-clinical cases of vCJD, then this will be of some concern, as the prevalence is much higher than expected from the observed incidence of clinical cases, either indicating a future increase in numbers of vCJD cases or a significant number of individuals with a 'carrier state' [40]. In the latter context, it is of interest to note that inoculation of the BSE agent into transgenic mice which express only the human PrP gene with methionine homozygosity at codon 129 has revealed a high incidence of sub-clinical infection [41]. In vCJD, immunohistochemical accumulation of PrP correlates with the presence of protease-resistant PrP, as determined by western blot examination [22] and infectivity [42]. Individuals with sufficient PrP accumulation to be detected by immunohistochemistry may therefore pose a health risk to others by causing iatrogenic spread via surgical instruments, blood transfusion or organ donation. Infectivity is not fully inactivated by autoclaving [43] and CJD has been transmitted by re-use of surgical instruments [44], although this risk is likely to be small (http://www.doh.gov.uk/cjd/consultation).

However, there has been a recent increase in concern about surgical transmission of CJD, following the demonstration of low levels of PrP^{Sc} in the skeletal muscle and spleen of some patients with sporadic CJD [45,46] and epidemiological studies that have shown an increased incidence of sporadic CJD following surgical procedures [47,48]. Abnormal PrP has not yet been demonstrated in the blood of patients with vCJD [17], but the most sensitive test for infectivity remains intra-species inoculation and data from sheep infected with BSE indicate that blood-borne transmission is possible [49]. A recent case of vCJD occurring in an individual 6 years after receiving a blood transfusion from a patient who later developed vCJD suggests that human blood is also able to transmit the disease [50]. Our findings therefore reinforce the importance of recent steps taken by the Department of Health to reduce these potential risks, which include the leucodepletion of all UK-sourced blood and the introduction of more stringent decontamination procedures for surgical instruments.

The incubation period of vCJD is not known and although numbers of cases are currently in decline, the possibility of further rises cannot be excluded. The average incubation period of kuru and iatrogenic CJD following peripheral inoculation has been estimated to be about 12 years, with some cases of kuru occurring more than 40 years after the cessation of cannibalism [44,51], but these diseases did not have to cross a species barrier. Data from a geographically associated cluster suggested that they resulted from exposure to BSE prior to 1986 (http://www.leicsha.org.uk/Publics/cjdrep.pdf), indicating an incubation period for these cases of 10–16 years.

Our study has demonstrated how a better understanding of the pathology of vCJD has allowed the investigation of an important epidemiological question about this disease using archival tissue collections. However, the techniques used in our study have been limited to immunohistochemistry, because of the use of formalin-fixed tissue sections, and by the study design, which prevents return to a positive tissue sample for further verification. These factors have limited the interpretation of our findings. However, we believe that they are of some concern and require urgent further investigation by prospective screening of tissue from tonsillectomies. By analysing fresh tissue, samples could be tested with a sensitive assay that allows for automation [17] and positive findings could be reliably confirmed by transmission studies. However, about half of tonsillectomies are performed on children under 10 years of age, so many individuals undergoing this procedure will soon have had little or no exposure to BSE and therefore the window of opportunity for such a study will diminish over time.

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