Rapid Testing for Creutzfeldt-Jakob Disease in Donors of Cornea

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Background. Creutzfeldt-Jakob disease (CJD) has been accidentally transmitted by contaminated corneal transplants. Eye donors are not ordinarily tested for CJD, in part because an easy test is not available. We propose a relatively simple postmortem procedure to collect brain samples without performing full autopsy and show that a test currently marketed for veterinary diagnosis would offer an effective screening test. **Methods.** We selected 6 brains from confirmed cases of human sporadic CJD and sampled each in triplicate (18 specimens), 28 control brains of individuals with non-CJD neurodegenerative diseases and 10 normal brains. We also applied a procedure involving retro-orbital puncture after enucleation and biopsied the frontal lobes and optic nerves of a macaque experimentally infected with variant CJD. All samples were tested with the IDEXX HerdChek BSE-Scrapie Ag Kit to detect the abnormal prion protein, PrP^{TSE}. **Results.** The test discriminated between control and CJD-infected brains. All 18 infected brain samples diluted to 0.1%, except one, showed signals above cutoff, and a number of samples were reactive at even higher dilutions. These results suggest the test could detect the low concentrations of PrP^{TSE} probably present in brains of donors at early stages of CJD. Our collection procedure obtained sufficient macaque brain and optic nerve tissues to detect PrP^{TSE}. **Conclusions.** We showed that a commercial test combined with rapid sample collection might offer a practical solution to screen brains of cornea donors for evidence of CJD. Such a test might enhance safety of corneal transplants and some other tissue-derived products.

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Creutzfeldt-Jakob disease (CJD) is a transmissible spongiform encephalopathy (TSE) or prion disease.^{1,2} The origin and incubation period of naturally acquired sporadic CJD (sCJD) remain unknown. No validated test identifies individuals infected with the agent causing sCJD before onset of overt illness. Diagnostic tests rely primarily

on postmortem examination of brain tissue for histological and biochemical abnormalities typical of CJD. The gold standard test is detection of protease-resistant abnormal prion protein, PrP^{TSE}, by Western blot (WB), although other methods such as detection of 14-3-3 protein in cerebral spinal fluid and, more recently, detection of PrP^{TSE} by Real-Time Quaking-Induced Conversion are also used by the US National Prion Disease Pathology Surveillance Center. More than 460 cases of iatrogenic CJD have been reported, most caused by exposure to tissues of undiagnosed CJD-infected donors.² Silent incubation periods of iatrogenic CJD often last many years. At least 6 iatrogenic transmissions of CJD from corneal transplantation have been confirmed or suspected worldwide.²⁻⁵ Iatrogenic CJD attributed to human cadaveric dura mater grafts and pituitary hormones has been even more common.²

Over 40 000 corneas are transplanted yearly in the United States.⁶ In many countries, including the United States, eye donors are “screened” by medical history to identify those with CJD risk factors and tested for evidence of several communicable disease agents.^{7,8} Review of medical history should exclude demented donors and some others at increased CJD risk.⁷ Donors are not tested for CJD for several reasons: no marketed human CJD screening test, low prevalence of CJD, and low autopsy rate. A prospective study testing brains of 1142 cornea donors by WB detected no CJD⁹ — a result not unexpected because of CJD relatively low prevalence (Centers for Disease Control and Prevention estimated lifetime risk for sCJD about 1 in

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L.G. designed the study, supervised the research, prepared and formatted figures and wrote the article. A.S. conducted the research, prepared figures and participated in the writing and reviewed the article. K.L.M. conducted early studies with IDEXX test that led to the work submitted; prepared figures and reviewed the article. J.C. conducted the macaque necropsy, removed tissues for testing and reviewed the article. D.M.A. designed the study, contributed to the writing, reviewed and edited the article.

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10 000¹⁰); the study concluded that CJD testing of cornea donors was not cost-effective. Another investigation looked at the feasibility of testing tonsil tissue to identify deceased tissue donors with variant CJD (vCJD).¹¹ In most cases, tonsils and other lymphoid tissues of vCJD patients have contained abnormal prion protein, PrP^{TSE}, the target analyte of the assay used.¹² The authors concluded that tonsil was a suitable tissue for routine testing (though rigor mortis prevented collecting tonsils in some cases). However, in sCJD and familial CJD infections (much more common than vCJD) PrP^{TSE} has not been detected in tonsil. Brain tissue is probably the most appropriate tissue to test cornea donors for evidence of CJD.

We believe that logistical difficulties and time involved in postmortem CJD testing can be substantially reduced. We propose a relatively simple procedure to collect a small amount of brain that requires no conventional autopsy. A similar procedure has been used successfully to test animal brains for rapid diagnosis of rabies.^{13,14} Rapid detection of PrP^{TSE} follows brain biopsy.

MATERIALS AND METHODS

Sample Preparation and PrP Testing

Human brain tissues were frozen fresh at the time of autopsy and stored at -80°C in a National Institutes of Health archive previously described Brown et al 1994 and later at the FDA. We thawed and removed 3 small samples from each of 6 brains of histopathologically confirmed CJD cases (CJD1-CJD6, samples A, B, and C) using 4-mm diameter disposable biopsy punches with plungers (Integra Miltex, York, PA). Sampling was random with a specific effort to collect from areas of the cortex. We used the IDEXX HerdChek BSE-Scrapie Ag Kit (HC test) (Westbrook, ME) to detect PrP^{TSE} in the brain samples. Approximately 100 mg of tissue from each of the 18 specimens were placed into tared tubes (supplied with kit) preloaded with 1 mL of homogenization buffer and ceramic beads, yielding about 10% w/v brain homogenates. Tissues were homogenized by vigorously shaking the tubes twice for 1.5 min each time in a bead-beater (BioSpec Products, Bartlesville, OK), holding tubes on ice for 5 min between homogenizations. HC tests were conducted with duplicate aliquots of each sample according to the manufacturer's instructions. Briefly, in a microtiter plate, 50 µL of brain suspension were mixed with 50 µL of homogenization buffer and 25 µL of working plate diluent from the kit. A 100-µL aliquot of the mixture was transferred to an HC test antigen-capture 96-well plate (wells coated with a proprietary ligand that binds PrP^{TSE} but not normal PrP) and mixed for 1 hour at room temperature. All buffers and necessary reagents were provided in the test kit. Wells were washed extensively and incubated with conditioning buffer followed by antibody to PrP conjugated to horseradish peroxidase (HRP). PrP^{TSE} bound to the well was detected by addition of a HRP substrate and the reaction was stopped by adding dilute acid. Color in each well was measured at 2 wavelengths, 450 nm and 562 nm, with a microtiter plate reader; the difference between the 2 measurements was calculated and the average for duplicate aliquots reported as final result for each sample. HC tests took approximately 5 hours to perform overall; however, the manufacturer also provides a 90-minute protocol that we did not test. To assess assay specificity, we tested

brain samples from 28 individuals diagnosed with several non-CJD neurodegenerative diseases and 10 normal brains. These control brains were prepared as 10% suspensions in homogenization buffer as described and tested with no further dilution. HC kits also include internal positive and negative controls.

Collection and Testing of Brain and Optic Nerve Tissues From a Macaque Infected With vCJD

A rhesus macaque (DER9) inoculated intravenously (10 mL) and intraperitoneally (2 mL) with 1% macaque-derived vCJD brain homogenate succumbed to the disease 25 months postinoculation. This animal was part of a different study¹⁵ ongoing in our laboratory. At necropsy, we removed both eyes and sampled a portion of the optic nerve for testing. Next, a small pilot hole was drilled in the superior infra-orbital aspects of the frontal bone using a Dremel rotary tool with flex-shaft attachment and 1.5-mm cylindrical drill bit. We enlarged the hole with a conical bit to accommodate a 4-mm biopsy punch mentioned above and incised the dura. The biopsy punch was inserted through the aperture toward the base of frontal lobe and gently rotated it. The tissue removed was expelled and weighed. All tissues (optic nerve samples weighing 0.1 g and 0.09 g and brain samples 0.07 g and 0.08 g) were transferred to HC test kit tubes containing premeasured volumes of homogenization buffer to yield 10% homogenates. The optic nerve tissue was fibrous and more difficult to homogenize than the brain; we repeated the homogenization until no large pieces of tissue remained visible. The first attempt to detect PrP^{TSE} in optic nerve samples yielded only low assay signals. To improve sensitivity we repeated the PrP^{TSE} capture step in the same well adding fresh tissue homogenate for a total of 100 µL of optic nerve homogenate per well tested. The rest of the protocol was as described above.

RESULTS

We selected, as a representative test for PrP^{TSE}, the IDEXX HC test, a veterinary test widely used in Europe and in North America to identify animals with bovine spongiform encephalopathy (BSE) and scrapie.¹⁶⁻¹⁸ The HC test is a rapid enzyme-linked immunosorbent assay-like test using wells coated with proprietary affinity ligand that preferentially binds to PrP^{TSE} and not to normal PrP. The assay, unlike most PrP^{TSE} tests, requires no removal of normal PrP by pretreatment with proteinase K. Two studies comparing PrP^{TSE} assays concluded that the HC test was among the more sensitive and specific tests for BSE currently on the market.^{16,17}

We used the HC test to assay 18 specimens from 6 sCJD brains suspended to 1% (w/v) in 10% normal human brain homogenate to maintain a constant content of brain proteins and 38 10% w/v control brains. Figure 1 displays readouts for all control samples, giving a mean optical density (OD) value 0.16 ± 0.05 OD₄₅₀₋₅₆₂. The test was 100% specific (no false positive) with OD signals tightly distributed around the mean. Test results with 1% CJD brain homogenates (Figure 1) revealed a close distribution of values around 3.2 OD₄₅₀₋₅₆₂. Sample CJD6-B was an outlier, giving a positive signal of 0.8 OD₄₅₀₋₅₆₂, still above the cutoff but much lower than signals from all other CJD samples including the 2 other specimens from different areas of the same brain (CJD6-A and CJD6-C), suggesting either

FIGURE 1. HC test signal distributions for normal control human samples (gray bars) and for CJD-infected samples (black bars). Normal samples included 10 healthy brains and 28 brains from patients with a variety of non-TSE degenerative neurological diseases: 8 with Lewy Body dementia, 7 with Alzheimer disease, 5 with Parkinson disease, 4 with Pick disease and 4 with Huntington disease. All normal brains were tested as 10% w/v homogenates. All CJD-infected brains were tested as 1% brain homogenates.

technical error or that CJD6-B was sampled from a site poor in PrP^{TSE}. That discrepancy aside, Figure 1 shows that replicate HC tests clearly discriminated between uninfected and CJD-infected samples.

When properly screened, individuals with overt CJD—source of all our CJD samples—should be ineligible to donate tissue because of dementia.⁷ The more likely danger comes from donors silently incubating CJD. Smaller amounts of PrP^{TSE} are probably present in brains during earlier stages of CJD incubation when individuals may still be asymptomatic.^{19,20} Since asymptomatic CJD-infected human brains are not obtainable, we modeled that scenario using serially diluted CJD brains. Eighteen 10% brain suspensions from the 6 CJD cases described above were diluted in 5 half-log₁₀ steps from 1% to 0.01% using 10% normal human brain homogenate as diluent. The dose-response curves for all 18 specimens are shown in Figure S1, SDC, (<http://links.lww.com/TP/B388>). These data are combined and summarized in Table 1, stratified by dilutions and analyzed with two cutoffs: 0.31 OD_{450–562} and 0.41 OD_{450–562} representing 3 times and 5 times the standard deviation above the mean, respectively. All 38 control brain samples were negative using both cutoff

values; such high specificity is an important advantage for a potential screening test. All infected samples diluted up to 0.1% were detected by the assay, with the exception of 1 outlier discussed above. At higher sCJD brain dilutions, 6 (33%) of 18 and 10 (55%) of 18 were not detected at dilutions 0.03% and 0.01% when using 0.31 OD_{450–562} cutoff. Slightly more samples fell below 0.41 OD_{450–562} cutoff. Despite brain-to-brain variability, the data suggest that in most cases the test discriminated an uninfected brain from a CJD brain even when diluted.

The test showed the same sensitivity with 0.1% homogenates of 4 CJD brains (3 sCJD cases and 1 vCJD case) provided by World Health Organization as candidate TSE biological reference materials²¹ (data not shown).

We also compared sensitivity of HC and WB assays (Figure S2, SDC, <http://links.lww.com/TP/B388>) using samples CJD6-A and CJD4-C. The limit of detection by WB was 1% brain suspension using a well-characterized monoclonal antibody 3F4, whereas HC detected signals above cutoff for both brain samples diluted up to 0.01%.

We assessed feasibility of a proposed tissue collection method that does not require full autopsy to sample brain tissue for sCJD screening. The procedure successfully removed sufficient brain and optic nerve tissues for testing from a macaque experimentally infected with vCJD. We tested 4 samples: optic nerve and brain tissues from left and right eyes. The results shown in Figure 2 demonstrate strong HC signals with optic nerve and brain tissues. The protocol for testing optic nerve was slightly modified to increase the volume tested (see Materials and Methods). Alternatively, the sensitivity of the methods would improve if 20% homogenates were prepared from optic nerves. The lower level of PrP^{TSE} in the optic nerve might either result from poor homogenization or reflect a true low concentration of PrP^{TSE} in this tissue (Figure 2).

DISCUSSION

Tissue banks do not currently test to exclude donors who might be incubating CJD. An effective test for PrP^{TSE} might sometimes enhance current practices, especially when a

TABLE 1.
Summary of HC test results with 18 serially diluted CJD brain samples and 38 negative controls

% Brain Homogenate	Cutoff	Cutoff
	0.31 OD _{450–562}	0.41 OD _{450–562}
1.0%	18/18	18/18
0.30%	18/18	17 ^a /18
0.1%	17 ^a /18	17 ^a /18
0.03%	12/18	13/18
0.01%	8/18	5/18
10% uninfected controls	0 ^b /38	0/38

For each CJD-infected specimen, we report the number of samples with OD above cutoff divided by the total number of samples tested.
^a The single false negative result was with CJD6-B.
^b Two samples were very close to the cutoff.

FIGURE 2. HC results with macaque DER9 optic nerve (ON) right (R) and left (L) and brain tissues from behind the right and left eyes. Optic nerve tissue had lower levels of PrP^{TSE} so double volumes were tested. Positive and negative run controls were provided as part of the HC test kit.

donor's antemortem mental status cannot be determined with confidence. Infectivity is consistently present in the brains of CJD-infected individuals^{22,23}; the optic nerve and retina are also infected and may contain detectable PrP^{TSE} protein.^{24,25} World Health Organization's most recent summary of the distribution of TSE infectivity in various tissues concludes that "In experimental models of TSE, the optic nerve has been shown to be a route of neuro-invasion, and contains high titers of infectivity."²⁶ In this work, we proposed to sample the region of the brain closest to the optic nerve—the frontal lobe—a region likely to contain sufficient PrP^{TSE} for detection. Our goal was to provide experimental evidence that brain tissue could be obtained postmortem without a full autopsy using a relatively simple procedure and that a commercial veterinary test, like the HC test, might be suitable to screen cornea donors for evidence of CJD. We also showed that the HC test was 100-fold more sensitive and faster than the gold standard WB for detecting PrP^{TSE} in postmortem brain samples. Other highly sensitive methods to amplify *in vitro* PrP^{TSE} such as Real-Time Quaking-Induced Conversion or Protein Misfolding Cyclic Amplification (PMCA) assays are also available.^{27,28} However, we did not select these methods because they are not commercially available.

The levels of PrP^{TSE} in the brains of presymptomatic individuals are not known and likely vary from case to case depending on disease status. To mimic this situation, we challenged the assay with decreasing concentrations of PrP^{TSE} from CJD-infected brains. We observed that, despite some variability, all brain homogenates, except one, diluted up to 0.1% were reactive in the HC test. Concentrations of PrP^{TSE} vary in different parts of the brain and retro-orbital puncture allows access to only a limited area. Thus, it would be safer in practice to sample both frontal lobes of a cornea donor, increasing the probability of detecting PrP^{TSE}.

Two control brains had OD values very close to the lower cutoff value analyzed (0.300 and 0.304) and that might have

been interpreted as weak CJD-positive results (false positive) triggering the exclusion of a donor. Thus, using the higher cutoff value of 0.41 OD has the advantage of yielding fewer false positives with the disadvantage that more infected samples with low levels of PrP^{TSE} (expected in some asymptomatic CJD-infected donors) might fall below the cutoff and be considered negative (false negative). Considering that CJD is a fatal and rare disease, it might be more prudent to set the assay cutoff at 0.31 OD to capture those brains with very low PrP^{TSE} concentrations (possible cases of presymptomatic donors) even though this strategy would inevitably exclude a few uninfected donors. However, the user of the assay will have to establish an appropriate cutoff (based on experimental testing) to minimize the number of excluded normal donors while optimizing the assay's specific reactivity.

We are aware of study limitations. The number of CJD samples available to test was too small to evaluate rigorously the performance characteristics of a test under field conditions. We have no access to autopsies of patients with CJD. Thus, we experimentally confirmed our proposed procedure by accessing the frontal-lobe cortex of a macaque dying with experimental vCJD. sCJD-infected human brain probably has levels of PrP^{TSE} similar to those in vCJD-infected macaque brain. All macaque samples were positive with the HC test including the optic nerves. Ideally, CJD screening would test brain tissue; however, our results indicate that optic nerve could serve as a useful alternative to brain with the advantage of easier access to screen cornea donors. We believe that sensitive assays for PrP^{TSE} offer a potential improvement over the current situation in which no CJD testing is conducted. Various options for implementation of this test to screen cornea donors could be considered: for example, universal testing versus targeting donors with risk factors for CJD or even possibly pooling several brain homogenates (though this option would reduce sensitivity.)

The HC test and other currently marketed TSE tests are veterinary diagnostics; they are neither intended for use with

nor optimized to test human tissue samples. We cannot endorse the off-label use of the HC test or any other commercial animal TSE test with human tissues. We acknowledge that a small frontal biopsy offers only a limited substitute for a complete evaluation of the brain in accurate postmortem diagnosis of neurological diseases. This study was intended simply to demonstrate proof of concept—that rapid postmortem testing for PrP^{TSE} using very small samples of brain tissue should be possible without full autopsy using technology already developed. In some situations, PrP^{TSE} testing might add to the safety of cornea transplantation already achieved by donor selection.

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