Immunoquantitative PCR for Prion Protein Detection in Sporadic Creutzfeldt–Jakob Disease

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Background: The most common human prion disorder is Creutzfeldt–Jakob disease (CJD); it includes sporadic, familial, iatrogenic, and variant subtypes. Diagnostic tests aim at detection with the highest specificity of very small deposits of abnormal prion protein (PrP).

Methods: We used immunoquantitative PCR (iqPCR) to detect proteinase K–resistant PrP (PrPRes) in tissue from the middle frontal gyrus of 7 patients with sporadic CJD and 7 non-CJD cases. We compared iqPCR with routine optimized ELISA, Western blotting, and immunohistochemical analyses.

Results: The 4 methods showed similar 100% sensitivity and specificity for the diagnosis of CJD. Along with high specificity, however, iqPCR had a threshold for PrPRes detection at least 10-fold lower than that of the classic ELISA.

Conclusions: iqPCR is a new method for PrPRes detection that combines 100% specificity with a detection threshold at least 10-fold lower than classic techniques. This method may improve the detection of minute PrPRes deposits in tissues and body fluids and thus be useful for diagnostic and sterilization applications.

Transmissible spongiform encephalopathies (TSEs)⁴ are disorders that involve proteins called prions (1). The cellular prion protein (PrP⁵) is a 33- to 35-kDa glycoprotein attached to the cell membrane by a glycosylphosphatidylinositol anchor; it is produced in most cell types, mainly by neurons (2). Pathogenic PrP results from a posttranslational modification of the cellular template (3). This nonconventional transmissible agent accumulates in the central nervous system (CNS), where it is associated with spongiosis, gliosis, and neuronal loss. It has a high β-sheet content compared with PrP⁶, which has more α-helices (4). This conformational change of α-helices into β-sheets confers particular physicochemical properties to the pathogenic PrP, such as solubility in nonionic detergents and partial proteinase K (PK) resistance (5). Detection of PK-resistant PrP (PrPRes) is the basis for TSE diagnostic tests.

Prion diseases are fatal neurodegenerative disorders. Clinical features include dementia, cerebellar ataxia, motor dysfunction, and behavioral changes. Among animal prion diseases, bovine spongiform encephalopathy (BSE) has been studied extensively. This disease reached epidemic proportions in the United Kingdom in the 1990s and threatens human health with the emergence of a variant form of Creutzfeldt–Jakob disease (vCJD) (6). Other human TSEs include sporadic and iatrogenic forms of CJD, Gerstmann–Sträussler–Scheinker syndrome, kuru, and fatal familial insomnia (7). The sporadic cases represent 85% of all prion diseases and are thought to be caused by spontaneous conformational changes of PrP. Familial forms are inherited in an autosomal dominant mode through germline mutations of the PrP gene, PRNP. Iatrogenic forms have occurred as a result of exposure to contaminated neurosurgical instruments, dura mater grafts, or transfer of human cadaver pituitary hormones (8). Kuru presented as an epidemic in Papua, New

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4 Nonstandard abbreviations: TSE, transmissible spongiform encephalopathy; PrP⁵, cellular prion protein; CNS, central nervous system; PK, proteinase K; PrPRes, PK-resistant prion protein; BSE, bovine spongiform encephalopathy; vCJD, variant Creutzfeldt–Jakob disease; iqPCR, immunoquantitative PCR; IHC, immunohistochemistry; PBS, phosphate-buffered saline; Ct, cycle threshold; and PrP⁶, prion protein–scrapie form.
Guinea, in the 1960s and has been linked to cannibalistic rituals. vCJD is zoonotically related to BSE (9, 10). An allelic polymorphism of codon 129 of human PRNP modulates disease susceptibility and clinicopathologic phenotype in CJD (11).

Detection of CJD is not possible at the preclinical stage. In symptomatic patients, clinical presentation, electroencephalographic features, brain imaging techniques (12), and 14-3-3 protein analysis in cerebrospinal fluid (13) may indicate CJD. Definitive diagnosis requires neuro-pathologic examination of the brain (14). The recent observation of an increase in sporadic CJD incidence and reports of possible blood transmission of vCJD (15) are strong incentives for the development of highly sensitive methods to detect PrPRes.

In this study, we assessed the sensitivity and specificity of immunoquantitative PCR (iqPCR) for the detection of PrPRes in the brains of CJD patients and compared sample analysis results obtained by iqPCR, immunohistochemistry (IHC), ELISA, and Western blotting.

**Materials and Methods**

**Case selection**

At postmortem examination, brain tissue samples were obtained from 7 patients with sporadic CJD and 7 control individuals. Neuropathologic diagnosis of CJD was based on extensive microscopic review of frontal, temporal, parietal, and occipital cortices; the cingulum, hippocampus, and amygdala; central gray matter; subthalamic nuclei; the brainstem; and the cerebellum. We selected 7 cases with characteristic clinical, histologic, and IHC features of sporadic CJD. Patient age at the time of death ranged from 57 to 81 years (mean, 68 years). Five patients presented with rapidly progressive dementia, 1 with cerebellar ataxia (CJD), and 1 with paresis and paresthesia of the left leg (patient CJD3). All patients later developed myoclonic movements and had abnormal electroencephalograms with periodic sharp wave complexes. There was no family history of demential disorders. Neuropathologic features were characteristic of spongiform encephalopathy, various degrees of spongiform changes, neuronal loss, and gliosis (14). In all 7 cases, IHC analysis with 3 monoclonal antibodies (3F4, KG9, and 4F7) confirmed the presence of PrPRes deposits, a finding that fulfills current neuropathologic criteria for definite CJD diagnosis (14, 17). The 7 control individuals included 4 patients with neurodegenerative brain diseases and 3 patients with healthy brain histology who died of nonneurologic disorders. This study was approved by the Ethical Committee of the Faculty of Medicine of the University of Liège. The clinical, histologic, and immunohistologic data are summarized in Table 1.

### Table 1. Histologic and immunohistologic data for study patients.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, years</th>
<th>Sex</th>
<th>Neurologic presentation</th>
<th>Final (postmortem) diagnosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>71</td>
<td>M</td>
<td>No neurologic disease</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>C2</td>
<td>74</td>
<td>M</td>
<td>No neurologic disease</td>
<td>Esophageal squamous cell carcinoma</td>
</tr>
<tr>
<td>C3</td>
<td>15</td>
<td>M</td>
<td>No neurologic disease</td>
<td>Sudden death (cardiac arrhythmia)</td>
</tr>
<tr>
<td>C4</td>
<td>82</td>
<td>M</td>
<td>Dementia</td>
<td>FTD-MNDb</td>
</tr>
<tr>
<td>C5</td>
<td>81</td>
<td>F</td>
<td>Dementia</td>
<td>Alzheimer disease</td>
</tr>
<tr>
<td>C6</td>
<td>68</td>
<td>M</td>
<td>Dementia</td>
<td>Alzheimer disease</td>
</tr>
<tr>
<td>C7</td>
<td>51</td>
<td>M</td>
<td>Dementia</td>
<td>FTDP-17</td>
</tr>
<tr>
<td>CJD1</td>
<td>73</td>
<td>F</td>
<td>Rapidly progressive dementia</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD2</td>
<td>57</td>
<td>M</td>
<td>Rapidly progressive dementia</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD3</td>
<td>62</td>
<td>M</td>
<td>Paresis and paresthesia in left lower limb</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD4</td>
<td>81</td>
<td>M</td>
<td>Cerebellar ataxia</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD5</td>
<td>71</td>
<td>F</td>
<td>Rapidly progressive dementia</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD6</td>
<td>70</td>
<td>M</td>
<td>Rapidly progressive dementia</td>
<td>Sporadic CJD</td>
</tr>
<tr>
<td>CJD7</td>
<td>63</td>
<td>F</td>
<td>Rapidly progressive dementia</td>
<td>Sporadic CJD</td>
</tr>
</tbody>
</table>

*Clinical data for diagnosis: for Alzheimer disease, diagnosis was based on CERAD criteria (26); for sporadic CJD, diagnosis was based on neuropathologic criteria updated by Budka et al. (17) and used by the WHO (reviewed by Budka (14)); diagnoses of frontotemporal dementia with motor neuron disease and frontotemporal dementia with parkinsonism linked to chromosome 17 were based on criteria proposed by the Work Group on Frontotemporal Dementia and Pick’s Disease (27).

b FTD-MND, frontotemporal dementia with motor neuron disease; FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17.
137 mmol/L NaCl, 3 mmol/L KCl), as described previously (20). Sections were blocked in normal rabbit serum (1:10 dilution; Vector) for 1 h, rinsed, incubated overnight at 4 °C with 3 primary monoclonal antibodies against PrP [3F4 (residues 109–111 of the human form; 1:50 dilution; Dako), KG9 (residues 140–180 of the human form; 1:250 dilution; TSE Resource Center), and 4F7 (residues 140–160; 1:1000 dilution; Roboscreen)], and then incubated with secondary antibodies (ENVISION; Dako) for 30 min. Immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB+, Dako). Negative controls were incubations in which the primary antibody was omitted.

**ELISA, Western Blots, and iqPCR**

**Sample preparation.** For ELISA, Western blots, and iqPCR, human PrP was extracted by routine optimized purification (Bio-Rad Platelia BSE Kit; cat. no. 355-1102) from frozen brain tissue blocks representative of the middle frontal gyrus. Briefly, nervous tissue (±350 mg) was homogenized for 45 s, and 500 μL of this homogenate was treated with PK for 14 min at 37 °C in buffer A. After the reaction was stopped and 500 μL of buffer B was added, tubes were centrifuged for 5 min at 20 000g. Immunodetection was performed on pellets previously resuspended in buffer C1 and heated 5 min at 100 °C in buffer C1. The positive control was a recombinant cellular human prion protein (Roboscreen).

For ELISA, Western strips, and iqPCR, human PrP was extracted by routine optimized purification (Bio-Rad Platelia BSE Kit; cat. no. 355-1102) from frozen brain tissue blocks representative of the middle frontal gyrus. Briefly, nervous tissue (±350 mg) was homogenized for 45 s, and 500 μL of this homogenate was treated with PK for 14 min at 37 °C in buffer A. After the reaction was stopped and 500 μL of buffer B was added, tubes were centrifuged for 5 min at 20 000g. Immunodetection was performed on pellets previously resuspended in buffer C1 and heated 5 min at 100 °C in buffer C1. The positive control was a recombinant cellular human prion protein (Roboscreen).

**ELISA.** Plastic well surfaces of Immunostrips (Maxisorp; Nunc) or Robostrips® (Roboscreen) were precoated with 10 mg/L monoclonal antibody 1E5 (Roboscreen) at 6–8 °C overnight. The next day, the wells were emptied, washed 3 times (washing buffer: 50 mmol/L Tris, 150 mmol/L NaCl, 0.5 mL/L Tween 20), blocked for 1 h with 200 μL of blocking buffer (washing buffer containing 10 g/L bovine albumin), and rinsed again. The precoated Immunostrips and Robostrips were then sealed in flat bags and stored at 4–6 °C until use. The precoated, saturated strips were incubated with different dilutions (crude and 1:5, 1:10, 1:50, 1:100, 1:500, and 1:5000 dilutions) of human brain extract for 1 h at room temperature. The calibrator (human recombinant PrP) was incubated in the wells at concentrations from 1 mg/L to 1 ng/L. After a 1-h incubation with detection antibody, the strips were washed 3 times with PBS containing 1 mL/L Tween and 3 times with PBS containing 15 g/L bovine serum albumin. Briefly, recombinant streptavidin (Roche) was preincubated for 45 min at 4 °C with biotinylated reporter DNA in a 1:2 molar ratio (21, 22). The resulting streptavidin–DNA complex was then added to the wells and incubated for 30 min at room temperature. The strips were washed 5 times with PBS and 10 times with distilled water, and then were subjected to PCR. Amplification was carried out in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 25 μL of SYBR Green PCR Master Mix (Applied Biosystems), 0.3 μM each primer, and 19 μL of water in a total volume of 50 μL. The temperature program was as follows: 10 min at 95 °C, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min for the annealing and extension phases.

SYBR Green is a dye that gives a fluorescence peak when it is intercalated in double-stranded DNA. Amplification curves were analyzed with Sequence Detection System software (Applied Biosystems), and the baseline (threshold) was determined to avoid background signals. The intersection between this threshold and the amplification curve, the cycle threshold (Ct), was plotted on a graph vs the sample dilution.

The reporter double-stranded DNA (EMBL sequence accession no. AX133313) used is original in that it was built by association of 2 DNA fragments from eukaryotic
and prokaryotic origin (21, 22). The primers were designed with Primer Express Software, Ver. 1.0 (Applied Biosystems): forward primer, 5'-AACCCCTGACGAA-CATCTCA-3'; reverse primer, 5'-GCCGCAGTGTGA-TGGATAT-3'.

**Western blots.** For Western blots, after the purification protocol, samples were boiled in loading buffer (Bio-Rad) and subjected to electrophoresis in 12% Tris-glycine gels (10 μL of protein extract and 10 μL of 2×-concentrated loading buffer per well). Proteins were then blotted on polyvinylidene fluoride (PVDF) membranes that had been blocked overnight at 4 °C with the ECL™ Advance Western blotting Solution (Amersham Biosciences). Samples were incubated with biotinylated 4F7 (0.1 mg/L) in a solution containing 2 g/L ECL Advance Blocking Agent in Tris-buffered saline containing 1 mL/L Tween for 1 h at room temperature on an orbital shaker. After washing, the membrane was incubated with peroxidase-conjugated streptavidin (Dako; diluted 1:7500) for 1 ha t room temperature. Specific bands of prion proteins were revealed by ECL Advance Western blot detection reagents and visualized with a ChemiDoc digital imager (Bio-Rad).

**Results**

IHC

No PrP deposits were detected in the CNS tissue of the 7 control individuals. By contrast, in the tissue samples from the 7 patients with sporadic CJD, PrP Res deposition was demonstrated by incubation of monoclonical antibodies 3F4 and KG9 with sections from the primary visual cortex and cerebellar cortex.

Spongiform changes, neuronal loss, and gliosis were present in all frontal cortex samples from the 7 patients with sporadic CJD (CJD1 to -7), although with various intensities. As shown in Table 1, differences were mild in patient CJD3, moderate in patients CJD2, CJD4, CJD5, and CJD6, and severe in patients CJD1 and CJD7.

Using KG9, we identified PrP Res deposits in the frontal cortex of all 7 cases. This result is in accordance with the high sensitivity of KG9 reported by others (20). The pattern of PrP Res deposition was patchy/perivascular in patients CJD1, CJD5, and CJD6; the pattern was synaptic in the 4 remaining cases and particularly mild and focal in patients CJD2, CJD3, and CJD4. The antibody 3F4 detected PrP Res deposits in 6 of 7 cases, with the samples from patient CJD5 remaining negative. The pattern of deposition was patchy/perivascular in patients CJD1 and CJD6 and focal/synaptic in the 4 other cases (Fig. 2).

**ELISA**

As shown by the A450 nm values plotted vs sample dilution (Fig. 5), all control human brains were clearly negative: absorbance was close to 0. In samples from CJD brains, maximum absorbance values were variable (Fig. 5B). In general, the detection limit for PrP Res was between dilutions 1:10 and 1:100. The detection limit in this case was defined as the lowest concentration giving an absorbance 3 times higher than that given by the controls. As with

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**Table 2. Histologic and immunohistologic features of the frontal cortex in CJD patients.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Histology (spongiform changes)</th>
<th>KG9</th>
<th>3F4</th>
<th>4F7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CJD1</td>
<td>Severe</td>
<td>Patchy/perivascular ++++</td>
<td>Patchy/perivascular ++++</td>
<td>Patchy/perivascular ++++</td>
</tr>
<tr>
<td>CJD2</td>
<td>Moderate</td>
<td>Focal/synaptic +</td>
<td>Focal/synaptic +</td>
<td>Negative</td>
</tr>
<tr>
<td>CJD3</td>
<td>Mild</td>
<td>Focal/synaptic +</td>
<td>Focal/synaptic +</td>
<td>Focal/synaptic +</td>
</tr>
<tr>
<td>CJD4</td>
<td>Moderate</td>
<td>Focal/synaptic +</td>
<td>Focal/synaptic +</td>
<td>Focal/synaptic +</td>
</tr>
<tr>
<td>CJD5</td>
<td>Moderate with coalescent vacuoles</td>
<td>Patchy/perivascular +</td>
<td>Patchy/perivascular ++++</td>
<td>Patchy/perivascular ++++</td>
</tr>
<tr>
<td>CJD6</td>
<td>Moderate with coalescent vacuoles</td>
<td>Synaptic +++</td>
<td>Patchy/perivascular ++++</td>
<td>Patchy/perivascular ++++</td>
</tr>
<tr>
<td>CJD7</td>
<td>Severe</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a, mildly positive; +, moderately positive; + + +, strongly positive.
Western blotting, only low concentrations of PrPRes were retrieved from patient CJD4.

**Discussion**

In 2004, Cervenakova and Brown (23) reviewed screening tests for the diagnosis of prion diseases, including fluorescent correlation spectroscopy, Seprion ligand, conformation-dependent immunoassay, time-resolved fluorescence spectroscopy, and protein misfolding cyclic amplification. They also commented on immuno-PCR as a promising method for detecting PrPRes at very low concentrations (23).

In a previous study, we showed that bovine PrPRes can be detected with very high sensitivity by iqPCR (21). This technology, described previously by Zorzi et al. in patent WO0131056 (22), couples an antibody detection step similar to an ELISA with nucleic acid amplification by a real-time PCR procedure. The detection threshold of iqPCR is lower than classic ELISA for recombinant and infectious bovine PrP (21).
In the present study, we compared iqPCR with currently used routine methods, such as ELISA, Western blotting, and IHC, for the diagnosis of sporadic CJD cases. Each method showed 100% sensitivity and specificity in our series. With the iqPCR method, however, we detected the presence of PrP\textsubscript{Res} at concentrations at least 10-fold lower than with other methods. Like other PCR techniques, iqPCR is hampered by a nonspecific background signal (24). In our data, the background signal was probably generated by nonspecific binding of reagents to the well surface. We have optimized the blocking and washing steps, however, to maintain an acceptable signal-to-noise ratio and keep 100% specificity.

Because iqPCR combines 100% specificity with a detection threshold at least 10-fold lower than that of ELISA, iqPCR may improve the detection of minute amounts of PrP\textsubscript{Res} deposits in tissues and body fluids for diagnostic or sterilization applications.

Brain biopsies are rarely performed in CJD patients when diagnosis of treatable alternatives to spongiform encephalopathy are under serious consideration. In the series of 55 biopsies reported by Brown et al. (18), brain biopsy was diagnostic in 95% of cases later confirmed by autopsy. In our cases, changes indicating spongiform encephalopathy were present in the frontal cortex of all 7 cases, although with mild intensity in 1 case (CJD3). In another, smaller series, Mahadevan et al. (19), using antibodies KG9 and 3F4, detected PrP\textsubscript{Res} deposition in only 5 of 8 brain frontal biopsies from CJD patients. In our study, by combining the KG9 and 3F4 antibodies with PK treatment, we detected PrP\textsubscript{Res} deposits in 7 of 7 and 6 of 7 cases, respectively. In 4 cases (patients CJD2 to -5), however, deposits were mild and focal, with a synaptic pattern, and could have been missed on an infracentimetric biopsy. Western blotting was confirmatory in most of these cases but was inconclusive in 1 (CJD4), for which ELISA and iqPCR were clearly positive. The main limitation of iqPCR at this stage is the need for fresh or cryopreserved tissue. We are currently adapting the method for use on formalin-fixed, paraffin-embedded material. A recent report (20) suggests that well-preserved PrP\textsubscript{Res} can be retrieved in useful amounts from such formalin-fixed, paraffin-embedded material. The application of iqPCR to archival material would allow large retrospective studies.

A previous report has shown that macaques infected with extracts of BSE-infected brains have PrP\textsubscript{Res} in extra-CNS tissues, such as lymphoreticular tissue in the tonsils, Peyer’s patches, and the spleen (15). Moreover, Glatzel et al. (25), using a special phosphotungstate preconcentration procedure, showed that PrP\textsubscript{Res} is present in lymphoid tissue. We have recently confirmed that PrP\textsubscript{Res} can be detected in lymphoid tissue by iqPCR, and this opens up the possibility of using this method to detect prions in archival material.

### Table 3. Summary of the ELISA and iqPCR detection limits: Lowest dilutions that were above the cutoff values for each CJD patient.

<table>
<thead>
<tr>
<th>Case</th>
<th>CJD1</th>
<th>CJD2</th>
<th>CJD3</th>
<th>CJD4</th>
<th>CJD5</th>
<th>CJD6</th>
<th>CJD7</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>50</td>
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<tr>
<td>iqPCR</td>
<td>1000</td>
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<td>500</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>500</td>
</tr>
</tbody>
</table>
tion step, found PrP$\text{Res}$. in the spleen and muscles of some patients with sporadic CJD. These reports suggest that small PrP$\text{Res}$ deposits occur outside the CNS in primate and human TSEs, in both sporadic and variant (BSE-related) subtypes.

iqPCR may be useful to analyze extra-CNS tissue in which PrP$\text{Res}$ accumulates in prion diseases. As reported previously (23), immuno-PCR is a sensitive method that enables detection of small amounts of molecules, but it needs refinement. We have developed an iqPCR method with real-time analysis to improve the technique and avoid time-consuming post-PCR handling with real-time analysis to improve the technique and needs refinement. We have developed an iqPCR method enables detection of small amounts of molecules, but it needs refinement. We have developed an iqPCR method with real-time analysis to improve the technique and avoid time-consuming post-PCR handling (21). We intend in the future to couple the sensitivity of iqPCR with prion protein–scrapie form (PrP$\text{Sc}$) concentration steps based on phosphotungstic acid precipitation or other means to detect PrP$\text{Sc}$ in very low amounts.

The recently reported suspicion of transmission of vCJD by blood transfusion (16) demonstrates the need for detection methods allowing certification of totally effective decontamination procedures for transfusion material, as well as for neurosurgical and endoscopic instruments. Monitoring of sterilization effectiveness may represent another application of iqPCR.

In conclusion, we have developed iqPCR as a new technique for PrP$\text{Res}$ detection. In our study, iqPCR combined 100% specificity with a detection threshold at least 10-fold lower than that of ELISA. This new method may be useful for the detection of minute PrP$\text{Res}$ deposits in CNS and extra-CNS tissues, such as body fluids.

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References