Clinical Chemistry 51:9
1605–1611 (2005)

Molecular Diagnostics
and Genetics

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 (PrP^{Res}) 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 PrP^{Res} detection at least 10-fold lower than that of the classic ELISA.**

Conclusions: **iqPCR is a new method for PrP^{Res} 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 PrP^{Res} 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 (PrP^{C}) 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 PrP^{C}, 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 (PrP^{Res}) 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 human 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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Received February 23, 2005; accepted June 2, 2005.
Previously published online at DOI: 10.1373/clinchem.2005.050120

[4] Nonstandard abbreviations: TSE, transmissible spongiform encephalopathy; PrP^{C}, cellular prion protein; CNS, central nervous system; PK, proteinase K; PrP^{Res}, 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 PrP^{Sc}, 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 neuropathologic examination of the brain *(14)*. The recent observation of an increase in sporadic CJD incidence and reports of possible blood transmission of vCJD *(15, 16)* are strong incentives for the development of highly sensitive methods to detect PrP^{Res}.

In this study, we assessed the sensitivity and specificity of immunoquantitative PCR (iqPCR) for the detection of PrP^{Res} 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 PrP^{Res} 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.

IHC

For each case, sections (5-μm thickness) were cut from formalin-fixed, formic acid–treated, paraffin-embedded brain tissue representative of the middle frontal gyrus cortex, an area where diagnostic surgical biopsies for CJD have been performed *(18, 19)*; the primary visual cortex (area striata); and the hemispheric cerebellar cortex. Sections were hydrated with graded alcohols and incubated in H_2O_2 (3 mL/L) for 15 min. They were left in formic acid (96% by weight; Sigma) for 15 min, washed twice in running tap water (10 min each time), and autoclaved for 11 min at 126 °C in citrate buffer (pH 6) for antigen retrieval. PrP^{C} digestion was carried out for 5 min in 10 mg/L PK (Sigma) diluted in phosphate-buffered saline (PBS; 8 mmol/L $Na_2HPO_4 \cdot 2\ H_2O$, 1.5 mmol/L KH_2PO_4,

Table 1. Histologic and immunohistologic data for study patients.

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

[a] 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 20 000*g*. 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, 1:1000, 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 10 mg/L to 10 ng/L. The strips were washed with PBS and then incubated with the biotinylated detection antibody 4F7 (1 mg/L; Roboscreen) for 1 h at room temperature. The wells were again washed 3 times with PBS containing 1 mL/L Tween and 3 times with PBS containing 15 g/L bovine serum albumin. Peroxidase-conjugated streptavidin (Dako; diluted 1:7500) was added to each well and incubated for 30 min at room temperature. After 5 washes in PBS, the residual peroxidase activity was measured by means of chromogenic reaction with a solution containing equal amounts, by volume, of 3,3′,5,5′-tetramethylbenzidine and H_2O_2 (BD PharMingen). After incubation for 30 min in the dark at room temperature, the reaction was stopped by addition of 1 mol/L H_2SO_4. The absorbance of the reaction mixture was measured at 450 nm ($A_{450\ nm}$) with an automatic reader instrument (BioTek ELX800NB).

iqPCR. The iqPCR procedure (Fig. 1) was identical to that for ELISA up to the addition of the biotinylated detection antibody 4F7. iqPCR was carried out in Robostrips precoated and saturated with optimized buffer. At each step, the incubation volume was 50 μL/well, as it was for the ELISA. The calibrator was used in iqPCR at a dilution range 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

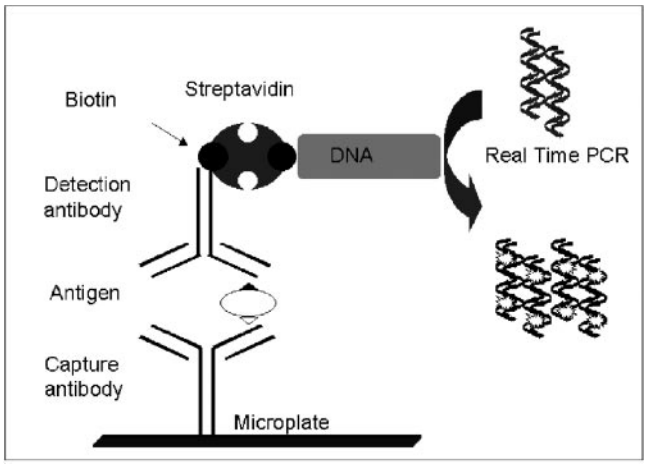

Fig. 1. Diagram of iqPCR.

and prokaryotic origin *(21, 22)*. The primers were designed with Primer Express Software, Ver. 1.0 (Applied Biosystems): forward primer, 5′-AAGCCTTGCAGGACATCTTCA-3′; reverse primer, 5′-GCCGCCAGTGTGATGGATAT-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 Blocking Agent (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 h at 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 monoclonal 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 2, changes 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/perivacuolar 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/perivacuolar in patients CJD1 and CJD6 and focal/synaptic in the 4 other cases (Fig. 2). Immunoreactivity for 4F7 was observed in 4 of 7 cases (CJD1, CJD3, CJD4, and CJD6).

WESTERN BLOTS

The different protein extracts from the 14 study patients were tested by Western blotting to check for the presence of PrP^{Res}. No signal was detected in non-CJD patients, whereas PrP^{Res} was detected in all CJD samples, with different loads, in correlation with the ELISA results. As shown in Fig. 3, high PrP^{Res} concentrations were observed in cases CJD1 and CJD2. The lowest PrP^{Res} load was observed in patient CJD4.

STANDARD IMMUNODETECTION

The human recombinant PrP calibrator was tested by ELISA and iqPCR in the same way as the brain samples. Calibration curves were plotted from a dilution range of the protein for both methods. The detection limits were ~1 μg/L (defined as 3 × the value for the control = 3 × 0.046) for the ELISA and ~100 ng/L for iqPCR. Detection limits of the iqPCR method were defined as the concentration giving a difference of 2.2 Ct (2 SD) lower than that given for the negative control without PrP (Ct = 30); the cutoff thus was 27.8 cycles (Fig. 4). The lower limit of detection for human recombinant PrP was therefore 10-fold lower than that of the ELISA technique.

ELISA

As shown by the $A_{450\ nm}$ 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

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

		IHC[a]		
Case	Histology (spongiform changes)	KG9	3F4	4F7
CJD1	Severe	Patchy/perivacuolar +++	Patchy/perivacuolar +++	Patchy/perivacuolar +++
CJD2	Moderate	Focal/synaptic +	Focal/synaptic +	Negative
CJD3	Mild	Focal/synaptic +	Focal/synaptic +	Focal/synaptic +
CJD4	Moderate	Focal/synaptic +	Focal/synaptic +	Focal/synaptic +
CJD5	Moderate with coalescent vacuoles	Patchy/perivacuolar +	Negative	Negative
CJD6	Moderate with coalescent vacuoles	Patchy/perivacuolar +++	Patchy/perivacuolar +++	Patchy/perivacuolar +++
CJD7	Severe	Synaptic ++	Focal/synaptic +	Negative

[a] +, mildly positive; + +, moderately positive; + + +, strongly positive.

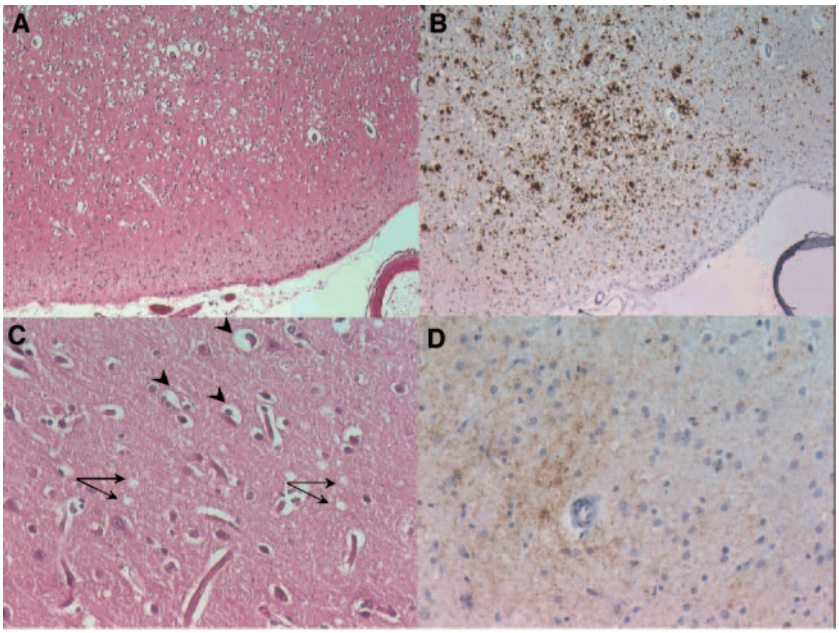

Fig. 2. Microscopic changes in the frontal cortex of CJD cases.

Tissue from patient CJD1 showed severe changes indicating spongiform encephalopathy after staining with hematoxylin and eosin (*A*) as well as abundant perivacuolar PrP^{Res} deposits after staining with antibody 3F4 (*B*; original magnification, ×50), leading to the obvious diagnosis of prion disease. In patient CJD4 (*C*), spongiform changes were mild/moderate, as visualized by staining with hematoxylin and eosin: true spongiosis (*arrows*) can be distinguished from vacuolar artifacts (*arrowheads*). Sparse deposits of PrP^{Res} (original magnification, ×200) were detected with antibody 3F4 (*D*).

Western blotting, only low concentrations of PrP^{Res} were retrieved from patient CJD4.

iqPCR

iqPCR results, plotted as the Ct vs sample dilution (Fig. 6) indicated that there was no significant nucleic amplification when the Ct was close to 40. For PK-treated non-CJD samples, the Ct was ~30 for all dilutions (Fig. 6A), which indicates that this is the background value. For all 7 CJD patients, the Ct was dose-dependent (Fig. 6B): the lower the PrP concentration, the higher the Ct. The detection limit of iqPCR was much lower than that of ELISA in all cases. In cases CJD1, CJD2, and CJD5, for example, PrP^{Res} was detected in the 1:1000 dilution; the iqPCR detection limit for PrP^{Res} thus was at least 10-fold lower than the detection limit of the ELISA for all patients (Table 3).

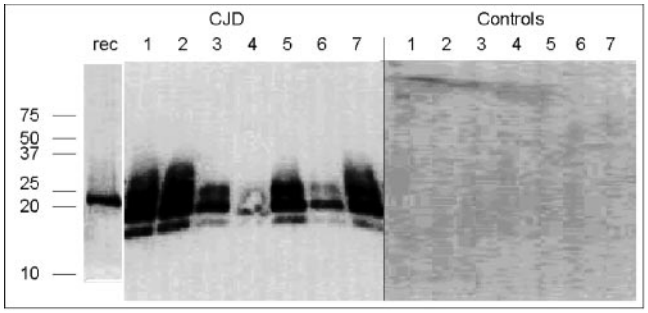

Fig. 3. Western blot of PK-treated brain samples from CJD cases and control cases (each well was loaded with the same amount of protein).

Recombinant human PrP (*rec*) was used as a positive control (100 ng). The immunoblots were analyzed by high-sensitivity ECL Advance using biotinylated anti-PrP monoclonal antibody 4F7 and peroxidase-conjugated streptavidin. Dual Color molecular mass markers (Bio-Rad), in kDa, are shown on the *left*.

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 PrP^{Res} at very low concentrations *(23)*.

In a previous study, we showed that bovine PrP^{Res} 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)*.

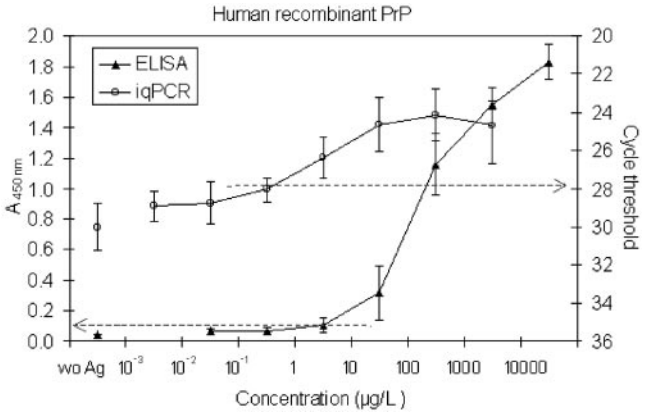

Fig. 4. Comparison of human recombinant PrP detection by ELISA (▲), with a cutoff at $A_{450\,nm}$ of 0.13, and iqPCR (○), with a cutoff at Ct 27.8.

Also shown is the negative control without antigen (*wo Ag*).

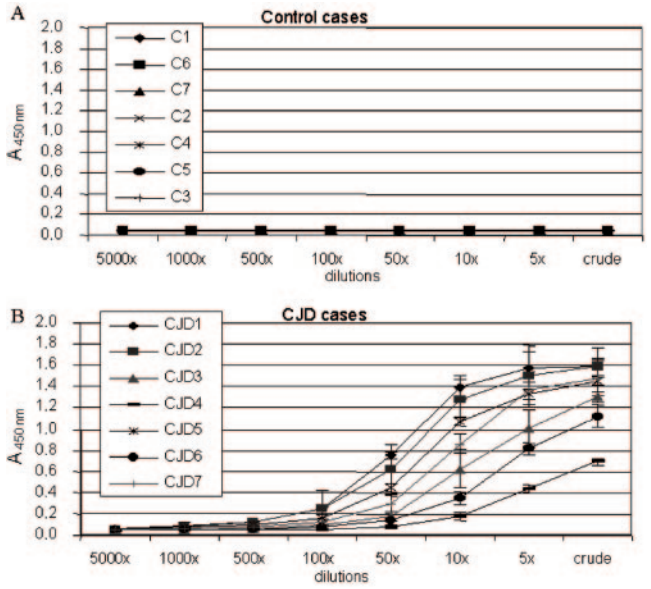

Fig. 5. Detection of PrP^{Res} by sandwich ELISA in control cases (*A*) and CJD patients (*B*).

$A_{450\ nm}$ is plotted against the protein extract dilutions. *Error bars* indicate SD from the means of 3 replicate experiments. The detection cutoff for the ELISA is $A_{450\ nm} = 0.13$.

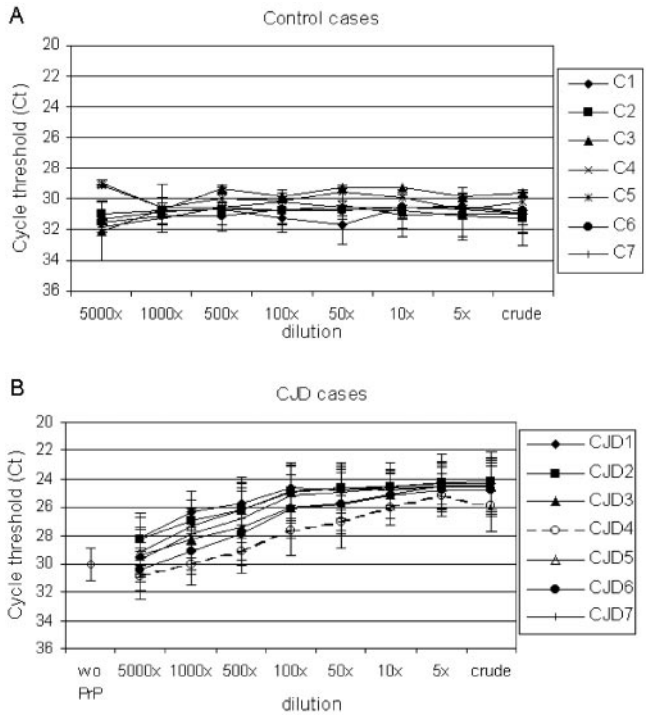

Fig. 6. Detection of human PrP^{Res} by iqPCR in control cases (*A*) and CJD patients (*B*).

The Ct is plotted vs the sample dilutions (*wo PrP*, negative control without PrP). *Error bars* indicate SD from the means of 3 replicate experiments. The detection cutoff for the iqPCR assay is Ct 27.8.

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^{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^{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^{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^{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^{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^{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 preconcentra-

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

	Case						
	CJD1	**CJD2**	**CJD3**	**CJD4**	**CJD5**	**CJD6**	**CJD7**
ELISA	100	100	50	10	100	10	50
iqPCR	1000	1000	500	100	1000	100	500

tion step, found PrP^{Res} in the spleen and muscles of some patients with sporadic CJD. These reports suggest that small PrP^{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^{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 *(21)*. We intend in the future to couple the sensitivity of iqPCR with prion protein–scrapie form (PrP^{Sc}) concentration steps based on phosphotungstic acid precipitation or other means to detect PrP^{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^{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^{Res} deposits in CNS and extra-CNS tissues, such as body fluids.

We gratefully acknowledge the financial support of this work by the Région Wallonne, Belgium (contracts RW 981/3799 and 14531).

References

1. Prusiner SB. Prions. Proc Natl Acad Sci U S A 1998;95:13363–83.
2. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982;216:136–44.
3. Weissmann C. Molecular genetics of transmissible spongiform encephalopathies. J Biol Chem 1999;274:3–6.
4. Liautard JP. Prion disease. The "prion" a remarkable infectious agent. J Soc Biol 1999;193:311–6.
5. Caughey B. Prion protein interconversions. Philos Trans R Soc Lond B Biol Sci 2001;356:197–202.
6. Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, et al. The same prion strain causes vCJD and BSE. Nature 1997; 389:448–50.
7. Harris DA. Prion diseases. Nutrition 2000;16:554–6.
8. Weller RO. Iatrogenic transmission of Creutzfeldt-Jakob disease. Psychol Med 1989;19:1–4.
9. Glatzel M, Ott PM, Linder T, Gebbers JO, Gmur A, Wust W, et al. Human prion diseases: epidemiology and integrated risk assessment. Lancet Neurol 2003;2:757–63.
10. Hill AF, Joiner S, Wadsworth JDF, Sidle KCL, Bell JE, Budka H, et al. Molecular classification of sporadic Creutzfeldt-Jakob disease. Brain 2003;126:1333–46.
11. Bratosiewicz-Wasik J, Wasik TJ, Liberski PP. Molecular approaches to mechanisms of prion diseases. Folia Neuropathol 2004;42(Suppl A):33–46.
12. Zerr I, Poser S. Clinical diagnosis and differential diagnosis of CJD and vCJD. With special emphasis on laboratory tests. APMIS 2002;110:88–98.
13. Muller WE, Laplanche JL, Ushijima H, Schroder HC. Novel approaches in diagnosis and therapy of Creutzfeldt-Jakob disease. Mech Ageing Dev 2000;116:193–218.
14. Budka H. Neuropathology of prion diseases. Br Med Bull 2003; 66:121–30.
15. Herzog C, Sales N, Etchegaray N, Charbonnier A, Freire S, Dormont D, et al. Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection. Lancet 2004;363:422–8.
16. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet 2004;363:417–21.
17. Budka H, Aguzzi A, Brown P, Brucher JM, Bugiani O, Gullota F, et al. Neuropathological diagnostic for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases). Brain Pathol 1995;5:459–66.
18. Brown P, Gibbs CJ Jr, Rodgers-Johnson P, Asher DM, Sulima MP, Bacote A, et al. Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. Ann Neurol 1994;35:513–29.
19. Mahadevan A, Shankar SK, Yasha TC, Santosh V, Sarkar C, Desai AP, et al. Brain biopsy in Creutzfeldt-Jakob disease: evolution of pathological changes by prion protein immunohistochemistry. Neuropathol Appl Neurobiol 2002;28:314–24.
20. Ritchie DL, Head MW, Ironside JW. Advances in the detection of prion protein in peripheral tissues of variant Creutzfeldt-Jakob disease patients using paraffin-embedded tissue blotting. Neuropathol Appl Neurobiol 2004;30:360–8.
21. Gofflot S, El Moualij B, Zorzi D, Melen L, Roels S, Quatpers D, et al. Immuno-quantitative polymerase chain reaction for detection and quantitation of prion protein. J Immunoassay Immunochem 2004;25:241–58.
22. Zorzi W, El Moualij B, Zorzi D, Heinen E, Melen L, inventors. Detection method by PCR. Patent WO 0131056, Belgium, 2001.
23. Cervenakova L, Brown P. Advances in screening test development for transmissible spongiform encephalopathies. Expert Rev Anti Infect Ther 2004;2:873–80.
24. McKie A, Samuel D, Cohen B, Saunders NA. Development of a quantitative immuno-PCR assay and its use to detect mumps-specific IgG in serum. J Immunol Methods 2002;261:167–75.
25. Glatzel M, Abela E, Maissen M, Aguzzi A. Extraneural pathological prion protein in sporadic Creutzfeldt-Jakob disease. N Eng J Med 2003;349:1812–20.
26. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991;41:479–86.
27. McKhann GM, Albert MS, Grossman M, Miller B, Dickson D, Trojanowski JQ. Clinical and pathological diagnosis of frontotemporal dementia: report of the Work Group on Frontotemporal Dementia and Pick's Disease. Arch Neurol 2001;58:1803–9.