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Rapid screening and confirmatory methods for biochemical diagnosis of human prion disease

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A B S T R A C T

Transmissible spongiform encephalopathies (TSEs) are characterised by accumulation of an abnormal isoform of prion protein (PrP^{sc}), mainly in the brain but also in various peripheral tissues. Home-made assays consisting of non-standardised protocols are used currently for laboratory diagnosis of human TSE. The purpose of the present study was to test the ability of two commercial assays, TeSeE™ CJD ELISA and TeSeE™ Western blot, to detect PrPsc in cerebral and lymphoid tissues of TSE patients. Both tests detected a PrPsc-significant signal in the brains of 54 affected patients and not in 51 controls, yielding 100% specificity and 100% sensitivity. Furthermore, three post-mortem spleens and two pre-mortem tonsils from three patients with variant Creutzfeldt–Jakob disease (vCJD) were detected correctly. The expected PrPsc molecular patterns were found in TSE patient brain tissue and in the tonsils and spleens of the three vCJD patients. In conclusion, these rapid and robust in vitro tools were suitable for routine human TSE diagnosis and characterisation. CJD could also be diagnosed during the patient's lifetime by detection of PrPsc in the tonsil. A diagnostic strategy associating TeSeE™ CJD ELISA screening to biochemical confirmation by TeSeE™ Western blot is proposed.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) include bovine spongiform encephalopathy (BSE), scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk and Creutzfeldt–Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann–Straüssler–Scheinker syndrome (GSS) in humans. In all these diseases, the conversion of the protease-sensitive cellular form of the prion protein (PrP^{c}) into a pathological isoform (PrP^{sc}) appears to be a key process in pathogenesis. The protease resistance of PrP^{sc} leads to the formation of a residual core of 27–30 kDa (PrP^{res}) after proteinase K (PK) treatment (Prusiner, 1998).

This property is used mainly in assay protocols for residual PrP^{res} detection by antibodies targeting the resistant core and/or the carboxylic moiety of PrP. For routine TSE diagnosis in animals, assays include the Western blot technique with or without concentration step and immunoassays such as LIA, IEA, ELISA or DELPHIA (Grassi et al., 2008). Development strategies for commercial tests are very different between TSE diagnosis in animals and humans. Whereas the BSE epidemic led to the development of rapid and robust commercial diagnostic assays for BSE and then scrapie and chronic wasting disease in veterinary programs, there are still no commercial kits for detecting PrP^{res} in CJD in humans. In most cases, laboratories use home-made methods based on Western blot which is the "gold standard" for biochemical diagnosis of CJD.

Various assay protocols for Western blot detection of PrP^{res} in CJD have been reported. The first described Western blot detection after PrP^{res} purification and concentration by an ultracentrifugation step (Brown et al., 1986). Direct PrP^{res} detection from 10% sample homogenates treated with PK then denatured in Laemmli buffer was recommended for its ease of use (Collinge et al., 1996; Parchi et al., 1996). Later, concentration steps using centrifugation, NaPTa precipitation, ultracentrifugation or streptomycin were introduced to enhance sensitivity, a key point in tissue other than brain (Favereaux et al., 2004; Lee et al., 2000; Quadrio et al., 2009; Wadsworth et al., 2001). All these methods, however, present certain disadvantages, lacking reproducibility, standardisation and robustness, and are time-consuming or make demands on operator experience to recover the ultracentrifugation pellet.

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Table 1
Diagnostic classification of TSE patients in retrospective and prospective studies.

Number of positive cases	Etiology	PrP^{res} molecular typing/prion protein gene mutation	Number of samples	Prior protein codon 129 polymorphism
Retrospective study				
6			3	MM ($n=1$)
		Type 1		MV ($n=1$)
				W ($n=1$)
	Sporadic CJD		3	MM ($n=1$)
		Type 2a		MV ($n=1$)
				VV ($n=1$)
6		Type 1/E200K	2	MM ($n=1$)
				MV ($n=1$)
		Type 1/D178NCJD	1	VV
	Genetic TSE	Type 2a/V2O3I	1	MM
		Type 2a/V210I	1	MV
		Type 2a/D178N FFI	1	MM
1	Latrogenic growth hormone CJD	Type 1	1	MM
1	Variant CJD	Type 2b	1	MM
Prospective study				
35			21	MM ($n=17$)
		Type 1		MV ($n=3$)
				nd ($n=1$)
	Sporadic CJD		11	MM ($n=1$)
		Type 2a		MV ($n=6$)
				VV ($n=4$)
		Type 1 + 2a	3	MV ($n=3$)
2	Genetic TSE	Type 1/E200K	1	MV
		Type2a/D178N FFI	1	MM
2	Latrogenic growth hormone CJD	Type 2a	1	MV
		Type 1 + 2a	1	nd
1	Variant CJD	Type 2b	1	MM

FFI: fatal familial insomnia; CJD: Creutzfeldt–Jakob disease; D: aspartic acid; E: glutamic acid; I: isoleucine; K: lysine; M: methionine; N: asparagine; V: valine.

In the animal field, 12 different tests were validated successively by the European Commission (Regulation (EC) N° 999/2001 of 22 May 2001, amended by Regulations N° 1053/2003 of 19 June 2003, N° 260/2005 of 16 February 2005 and N° 162/2009 of 26 February 2009 for the prevention, control and eradication of certain TSEs). In addition to its rapid tests for animal TSE screening (TeSeE™ SAP and TeSeE sheep and goat ELISA), Bio-Rad has also developed a commercial test for confirmation of suspected animal TSE (TeSeE™ Western blot). The maximum operating times from tissue homogenisation to plate reading in the ELISA protocol and to film development after immunoblotting is about 3 and 7 h, respectively. These relatively short times could enable further rapid and robust tools to be implemented in the diagnostic field. The present study assessed the performance and feasibility of these kits for detecting PrP^{res} in cerebral and lymphoid tissue in human TSEs.

2. Materials and methods

2.1. Post-mortem samples and pre-mortem tonsils

For the detection of PrP^{res} in brain, cerebral fragments (see Table 1) were treated in the Department of Neurochemistry BSL-3 laboratory dedicated to prion diseases at the Hospices Civils de Lyon. Necropsy brain tissues were collected from Lyon, Besançon and Marseille and treated according to the French legislation on ethical and technical issues.

For the retrospective study, 28 diagnosed patients (neuropathological lesions, immunohistochemistry and immunodetection of PrP^{res} on the frontal cortex by Western blot after ultracentrifugation concentration) were selected for their disease pattern: 14 patients without post-mortem CJD signs, 13 CJD and one fatal familial insomnia patient. These 14 TSE patients were selected so as to cover a large range of different diseases and/or various molecular types of PrP^{res} (Table 1). The non-TSE patients comprised 10 with neurodegenerative disease (Alzheimer's disease $n=6$, Lewy body dementia $n=2$, and Parkinson's disease $n=2$), three with vascular lesions and one with non-neurodegenerative disease. This cohort was studied as a control to assess the suitability of the TeSeE™ CJD ELISA and TeSeE™ Western blot assays for the detection of PrP^{res} in TSE-positive human cerebral tissue.

In a prospective study, 77 brains of suspected TSE patients were tested with the TeSeE™ CJD ELISA and TeSeE™ Western blot assays and, in parallel, results were validated with the reference Ultracentrifugation Western blot (UC-WB) technique used routinely in the laboratory. This cohort included 40 TSE patients described in Table 1, 1 of whom had fatal familial insomnia. Twenty of the 37 patients without post-mortem TSE signs had dementia, including 15 with Alzheimer's disease, 2 with Lewy body dementia, 3 with frontotemporal dementias and 1 with Parkinson's disease; the other 17 patients had no specific neurodegenerative lesions, although 2 presented vascular lesions. This cohort study was analysed to validate PrP^{res} detection in TSE-positive brains with the TeSeE™ CJD ELISA and TeSeE™ Western blot assays (Bio-Rad, Marnes-la-Coquette, France).

Furthermore, control and CJD human brain materials were obtained from the National Institute for Biological Standards and Control (NIBSC): two vCJD 129 Met/Met brains (NHBXO/0014, NHBYO/0003) and one CJD-negative 129 Met/Met brain (NHBZO/0005) (Minor et al., 2004).

For the detection of PrP^{res} in human spleen, three samples from patients diagnosed with vCJD were included after full necropsy, versus three without CJD.

For the detection of PrP^{res} in tonsil tissue, samples were obtained after diagnostic tonsillectomy for suspected vCJD in four patients. Two were classified as vCJD-positive after confirmation of diagnosis on necropsy brain tissue, whereas the other two were confirmed as vCJD-negative.

2.2. Preparation of sample homogenates

Sample tissues (350 mg brain or 200 mg lymphoid tissue) were homogenised with a TeSeE™ Precess 48™ (Bio-Rad, Marnes-la-

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Coquette, France) in grinding tubes containing ceramic beads and 1.4 mL 5% glucose solution. At least two different areas of each brain (frontal cortex, occipital cortex, striatum or cerebellum) were treated in independent homogenates for the prospective study. The homogenisation process was modified slightly for lymphoid tissue. After homogenisation, the weight/volume ratio was 20% for brain material and 12.5% for lymphoid tissue. One to three areas of spleen homogenate were also prepared and treated as separate homogenates. Since little material was available, 100 mg of one tonsil of a vCJD patient was diluted 2-fold in 100 mg tonsil from a non-CJD patient.

Two aliquots of sample homogenates, 250 μL and 500 μL, respectively, were calibrated and collected for the ELISA and Western blot assays, and frozen at −80 °C until analysis. For the prospective study, brain homogenates from necropsies of suspected CJD patients were divided into three aliquots before freezing. One aliquot was dedicated to the ultracentrifugation Western blot analysis for routine diagnosis. Calibrated homogenates were then treated with PK, and PrP^{res} was precipitated according to the manufacturer's user-manual recommendations described below.

2.3. Rapid test (TeSeE™ CJD ELISA)

The TeSeE™ CJD ELISA test (Bio-Rad, Marnes-la-Coquette, France) is a version of the TeSeE screening assay that is available commercially for the detection of TSEs in animal brain and peripheral tissues.

2.3.1. PrP^{res} purification

250 μL of the calibrated homogenates was digested for 10 min at 37 °C with 250 μL PK solution (including tensio-active agents). An adaptation for the PK concentration was needed for lymphoid tissue homogenates. Then 250 μL alcohol solution was added and each tube was centrifuged at 15,000 × *g* for 7 min. The pellet was dissolved at 100 °C for 5 min after addition of 25 μL buffer containing tensio-active agents. In cerebellar tissue, the pellet was sometimes too large and viscous to be treated and was therefore discarded.

2.3.2. PrP^{res} detection

At the end of the purification process, all samples were diluted in the kit diluent (R6 reagent). One hundred microliter of the diluted samples was then transferred into the wells of an ELISA microplate pre-coated with monoclonal antibody (3B5). After 75-min incubation at 37 °C, the microplate was washed 3 times and incubated with horseradish peroxidase (POD)-labelled monoclonal antibody (12F10) for 60 min at +2–8 °C. After 5 washing cycles, 100 μL substrate solution was added and colour development was obtained after incubation of 30 min at room temperature in the dark. Absorbencies at 450/620 nm were measured after stopping the reaction with 100 μL stop solution. The entire assay was performed with common commercial reagents and using the tube and microplate equipment recommended by the assay manufacturer.

For the retrospective study, all samples (negative and positive) were tested neat and diluted to 1:15 and 1:150 in the kit R6 sample diluent before being placed in the detection plate. In the second blind and prospective cohort, the first aliquot of calibrated brain homogenate was tested systematically neat and diluted at 1/150.

The various controls and human CJD brain materials from the National Institute for Biological Standards and Control (NIBSC) were 10% homogenised tissues in PBS in 0.25 M sucrose (Minor et al., 2004). Then, panel samples prepared with the two NIBSC vCJD brain homogenates spiked in a CJD-negative brain or tonsil homogenate were analysed to determine TeSeE™ CJD ELISA test sensitivity. Using negative brain or tonsil, dilution ranges were carried out from 167 μg to 2 μg CJD-positive tissue equivalent.

2.4. Western blot (TeSeE™ Western blot)

The TeSeE™ Western blot kit (Bio-Rad, Marnes-la-Coquette, France) is also currently marketed for confirmation of suspected animal TSE.

2.4.1. PrP^{res} purification

500 μL of the calibrated homogenates was treated as described previously but with 500 μL of a 5-fold concentration of PK solution and 500 μL of the same alcohol solution. An adaptation for the PK concentration was needed for the lymphoid tissue homogenates. After centrifugation, the pellet was solubilised by incubating at 100 °C for 5 min with 100 μL Laemmli buffer supplemented with beta-mercaptoethanol and SDS. All samples were then centrifuged at 15,000 × *g* for 15 min for clarification. The supernatants were transferred to new tubes. At this step, samples could be stored at −20 °C for 24 h.

2.4.2. PrP^{res} detection

Fifteen microliters of each sample, pre-heated at 100 °C for 4 min, were run on acrylamide gels for 90 min at 150 V (Mini-Protean 3, 1.5 mm cell and Power Pac 200 – Bio-Rad).

Proteins were transferred onto a PVDF membrane by 1-h electroblotting at 115 V in Tris-Caps buffer (Trans-Blot cell and Power Pac 200 – Bio-Rad). Following transfer, the membrane was prepared and blocked for 30 min (Bl kit reagent). It was then incubated for 30 min at room temperature with the monoclonal anti-PrP antibody Sha 31 (AbI kit reagent, Feraudet et al., 2005) under slow agitation on a Western blot Processor (Bio-Rad). After a brief rinse and 2 washing steps (5 and 10 min), the membrane was incubated for 20 min with a POD-labelled anti-mouse IgG goat antibody (AbII kit reagent). The ECL Western blotting detection reagents (Amersham) were used as substrate after 3 washing steps (respectively 5, 10 and 10 min). Results were visualised by exposure to ECL Hyperfilms (Amersham). Films were exposed classically for 15 min, but exposure time could be extended so as to optimise the signal. Molecular weights were determined with a protein standard (Magic Mark, Invitrogen, France).

In the retrospective study, positive and negative brain homogenates were first analysed neat. Secondly, positive ones (but excluding fatal familial insomnia) were diluted in Laemmli solution according to the optical density (OD) obtained with the TeSeE™ CJD ELISA test prior to loading on the gel. Lymphoid tissue homogenates were only tested neat.

For the prospective study, based on the retrospective study results and cut-off value, all samples negative on TeSeE™ CJD ELISA were tested neat with TeSeE™ CJD Western blot using the second brain homogenate aliquot, whereas positive samples were examined diluted in an adapted volume of Laemmli solution according to the TeSeE™ CJD ELISA OD value. Finally, TeSeE™ CJD Western blot test sensitivity was determined using the same NIBSC homogenates as for TeSeE™ CJD ELISA sensitivity.

2.5. Ultracentrifugation Western blot (UC-WB) protocol

UC-Western blot analysis was performed as described previously (Favereaux et al., 2004) with minor modifications (Quadrio et al., 2009). Briefly, 10% brain homogenates were obtained using glucose 5%. They were digested by proteinase K for 1 h at 37 °C and were further ultracentrifuged for 1 h 15 min at 800,000 RCF (Thermo Scientific Sorvall Discovery M150 SE, rotor S140AT, Thermo Fisher Scientific, Waltham, MA, USA) in N-lauryl sarcosyl. Pellets were resuspended and denatured. The supernatant was collected for Western blot analysis. Samples were run in 16% SDS-PAGE gels. After 9 h, diagnosis was obtained and a second Western

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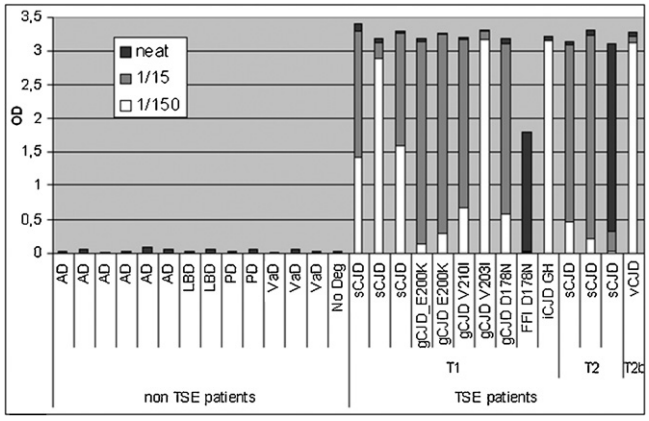

Fig. 1. Bar graph of cumulative optical density (OD) values from the frontal cortex homogenate analysis of the retrospective cohort. OD values higher than 3.0 are overflow. Neat, 1/15 and 1/150 brain homogenate OD values are shown (AD: Alzheimer's disease; LBD: Lewy body dementia; PD: Parkinson's disease; VaD: vascular dementia; No Deg: non-neurodegenerative disease; sCJD: sporadic Creutzfeldt–Jakob disease; vCJD: variant Creutzfeldt–Jakob disease; FFI: fatal familial insomnia; gCJD: genetic Creutzfeldt–Jakob disease; iCJD: iatrogenic Creutzfeldt–Jakob disease; GH: growth hormone).

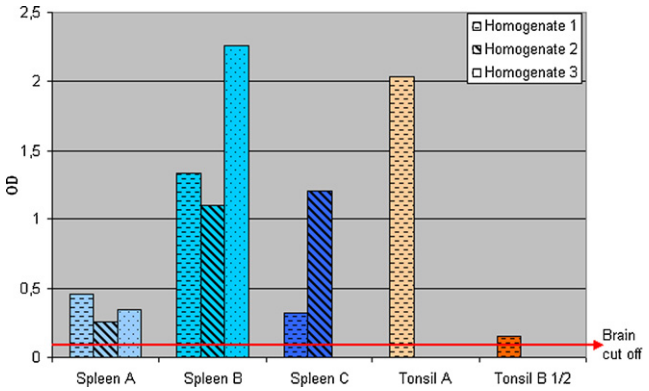

Fig. 2. TeSeE™ CJD ELISA results for peripheral tissue. Two or 3 different areas of spleen from 3 TSE patients were tested (homogenates 1, 2 and 3). Since there was only a small amount of tonsil material available, only one area was treated and, for one tonsil (B) obtained from a vCJD patient, 100 mg was diluted 2-fold in 100 mg of tonsil from a non-TSE patient. The red arrow represents the OD cut-off value from non-TSE brain homogenates (vCJD: variant Creutzfeldt–Jakob disease). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

blot was carried out on TSE-positive homogenates for PrP^{res} pattern characterisation.

3. Results

3.1. Retrospective study: suitability for human prion detection and confirmation of PrP^{res} in brain and lymphoid tissue

3.1.1. TeSeE™ CJD ELISA

3.1.1.1. Diagnostic sensitivity. Brain tissue: In the absence at the time of study of a predetermined cut-off value adapted for human brain tissue, a CJD and a TSE-negative population were compared.

From the cohort of 28 patients, the suitability of the TeSeE™ CJD ELISA test was confirmed, and an initial cut-off value was set (Fig. 1). For the negative samples ($n = 14$), tested neat, the mean and standard deviation (SD) were calculated at respectively 0.039 OD and 0.018 OD. OD values for positive samples were all between 1.796 and overflow (≥3.0 OD). The cut-off value of the test was then calculated by the mean plus three standard deviation of the negative sample: the cut-off value was 0.092 OD. According this value, the samples were classified as negative or positive.

In a similar approach, vCJD and non-CJD spleens and tonsils were also investigated by the rapid TeSeE™ CJD ELISA test (Fig. 2).

Tonsil: Two patients with vCJD and two CJD-negative patients were investigated. Absorbance values measured for the two vCJD samples were respectively 2.034 OD for vCJD case A (tested neat) and 0.151 OD for vCJD case B (tested diluted at 1/2). The two negative samples were clearly lower, at 0.023 OD and 0.024 OD.

Spleen: Three patients with vCJD and three CJD-negative patients were investigated. For positive samples, independent sample homogenates prepared from two or three tissue areas were tested. Spleen A was detected at 0.460, 0.260 and 0.342 OD while spleen C was detected at 0.323 and 1.206 OD. Spleen B was measured at 1.102, 2.259 and 1.339 OD. All non-CJD samples (Neg A to Neg C) gave lower ODs, from 0.018 to 0.022 OD, with a mean of 0.020 OD.

3.1.1.2. Detection limit. The detection limit estimated on NIBSC sample homogenates, prepared with the two NIBSC vCJD brain homogenates (NHBYO/0003 and NHBYO/0014) spiked in TSE-negative brain or tonsil homogenate, is recapitulated in Fig. 3. The dilution range was performed using negative brain or tonsil, and PrP^{res} was detected with good diagnostic discrimination in 19 μg CJD-positive brain equivalent. On analysis of the NIBSC vCJD samples, TeSeE™ CJD ELISA was able to detect PrP^{res} with the same level of optical density, regardless of matrix (brain or tonsil).

3.1.2. TeSeE™ Western blot

In this retrospective study, the expected typical three-band molecular pattern of PrP^{res} was identified correctly in all brain regions for the 13 CJD and one FFI patient, and not in the 14 non-TSE patients.

In peripheral tissue, typical CJD-positive patterns were also obtained with the TeSeE™ CJD Western blot test in lanes corresponding to CJD-positive spleen and diluted CJD-positive tonsil (Fig. 4, obtained after an exposure of 1 h).

The detection limit for analysis of the NIBSC vCJD samples by TeSeE™ CJD Western blot was 3 μg CJD-positive brain equivalent.

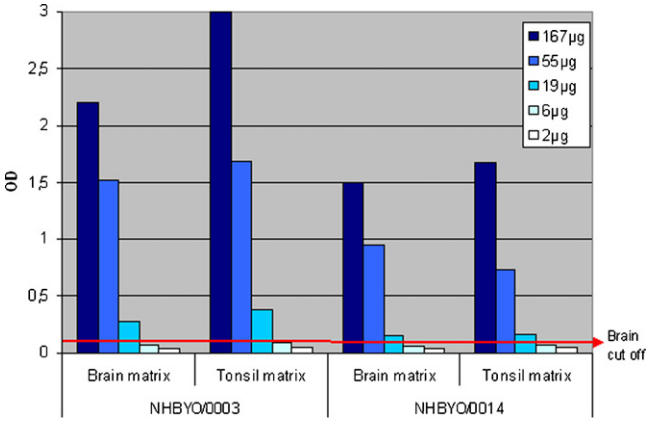

Fig. 3. The effect of NIBSC vCJD sample homogenate dilution in negative matrix for detection of PrP^{res} by the TeSeE™ CJD ELISA kit. Neat brain homogenates from NIBSC samples NHBYO/0003 and NHBYO/0014 were 10% homogenised tissues in PBS in 0.25 M sucrose. The first dilution was performed at 1:100 in either in negative brain homogenate or tonsil homogenate so as to obtain concentrations of 20% in brain matrix and 12.5% in tonsil matrix. The subsequent points were obtained by successive 3-fold incremental dilutions in R6 up to 1:8100. The red arrow represents the OD cut-off value from non-TSE brain homogenates (vCJD: variant Creutzfeldt–Jakob disease). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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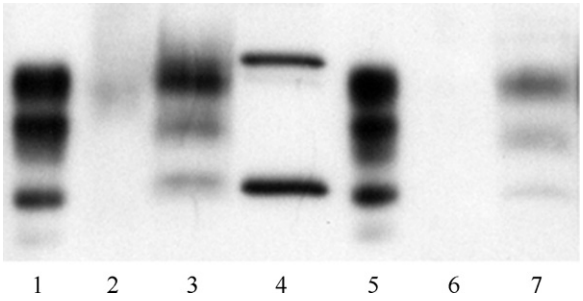

Fig. 4. TeSeE™ Western blot analysis from CJD or non-CJD lymphoid tissue homogenates (ECL, 1 h). Lanes 1 and 5: type 2b PrP^{res} control obtained with UC precipitation (brain). Lane 2: non-TSE spleen homogenate; lane 3: vCJD spleen homogenate; lane 4: molecular mass marker. The bands correspond to the molecular mass markers of 30 kDa and 20 kDa, respectively, from up to down. Lane 6: non-TSE tonsil homogenate; lane 7: vCJD patient, 100 mg diluted 2-fold in 100 mg tonsil from a non-TSE patient.

This test was able to detect PrP^{res} with the same band revelation intensity, regardless of matrix (brain or tonsil) (Fig. 5).

3.2. Prospective brain study

3.2.1. TeSeE™ CJD ELISA

Based on the results of the first study, brains from suspected CJD patients were tested prospectively as unknown samples in parallel to the routine UC-Western-blot technique. Of the 248 homogenates obtained from 77 patients, 10 cerebellum samples were too viscous to be tested (Table 2). Using the predetermined cut-off 0.092, 109 of the 238 remaining samples were negative and one weakly positive (OD 0.095) but not confirmed by an independent second run (OD 0.025). Finally, 110 were TSE-negative and 128 TSE-positive. Compared to the routine UC-WB and the neuropathological/immunohistochemistry (IHC) data, the sensitivity and specificity were both 100%. Thirty-seven negative (110 homogenates) and 40 positive patients (128 homogenates) were tested and identified correctly. Ninety-five percent of CJD-positive homogenates showed OD values better than 1.5. Five homogenates had OD values lower than 0.5 but nevertheless positive; four of these came from the neat FFI homogenates (0.15–0.45) and the other was an occipital cortex homogenate from type 2 PrP^{res} sCJD with optical density of 0.327, which was very low compared to the very high values (>3 OD) of the same patient from the other three brain areas tested. Finally, cut-off was determined on 128 TSE-negative homogenates. The final cut-off value was almost identical to that of the retrospective study, at 0.081.

3.2.2. TeSeE™ Western blot

The 248 homogenates were analysed in the light of the results obtained with TeSeE™ CJD ELISA, but blind to the data of routine UC-WB and neuropathological findings. One hundred and thirty-two neat homogenates (including 4 viscous homogenates) were found positive whereas 116 neat homogenates (including 6 viscous homogenates) were found negative. These data confirmed the TeSeE™ CJD ELISA data and were in agreement with the 100% specificity and sensitivity of the IHC and routine UC-WB method, leading to a classification of 37 TSE-negative and 40 TSE-positive patients (Fig. 6).

Table 2
Number of brain homogenates tested in the prospective study for each brain area. Brain homogenates are classified according to the retrospective OD cut-off value (OD = 0.092) and proved autopsy diagnosis.

CNS tested area	TSE patients		Non TSE patients	
Cerebellum	36 (+4 viscous)		31 (+6 viscous)	
OD > cut-off		36		0
OD < cut-off		0		31
Striatum	26		22	
OD > cut-off		26		0
OD < cut-off		0		22
Frontal cortex	38		37	
OD > cut-off		38		0
OD < cut-off		0		37
Occipital cortex	27		20	
OD > cut-off		27		0
OD < cut-off		0		20
Thalamus	1		0	
OD > cut-off		1		0
OD < cut-off		0		0
Total nb. of BH	128		110	
OD > cut-off		128		0
OD < cut-off		0		110

TSE: transmissible spongiform encephalopathy; Nb: number; OD: optical density; CNS: central nervous system; BH: brain homogenate.

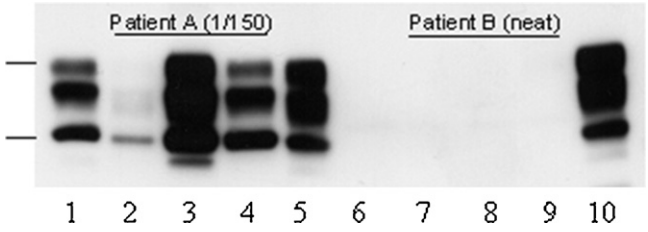

Fig. 6. TeSeE™ Western blot analysis from a sCJD patient (patient A) with type 1 PrP^{res} and a patient without TSE (patient B) (15 min in ECL). For each patient, 3 areas were treated: cerebellum (lanes 2 and 7), striatum (lanes 3 and 8), and frontal cortex (lanes 4 and 9). Lane 1: type 1 PrP^{res} control from sCJD. Lanes 2–4: patient A samples diluted in 1/150 in Laemmli buffer before loading on gel. Lanes 5 and 10: type 2a PrP^{res} control from sCJD. Lane 6: non-TSE control brain homogenate. Lanes 7–9: neat patient-B samples loaded on gel (sCJD: sporadic Creutzfeldt–Jakob disease).

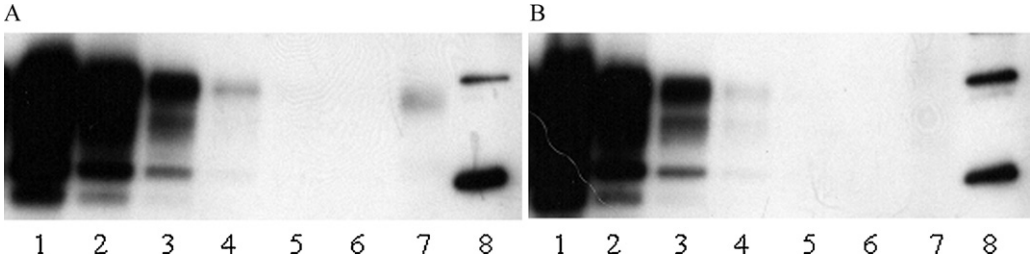

Fig. 5. TeSeE™ Western blot analysis from NHBYO/003 homogenate diluted in non-TSE brain (A) or in non-TSE tonsil (B) (ECL+, 30 min). Lane 1: NHBYO/003 homogenate diluted in 1/100 in non-TSE matrix (75 μg CJD-positive brain equivalent). Lanes 2–6: successive 3-fold Laemmli dilutions of NHBYO/003 homogenate diluted in 1/100 in non-TSE matrix (corresponding respectively to 25 μg, 8 μg, 3 μg, 1 μg and 0.3 μg of CJD-positive brain equivalent). Lane 7: neat non-TSE homogenate matrix. Lane 8: molecular mass marker. The bands correspond to the molecular mass markers of 30 kDa and 20 kDa, respectively, from up to down.

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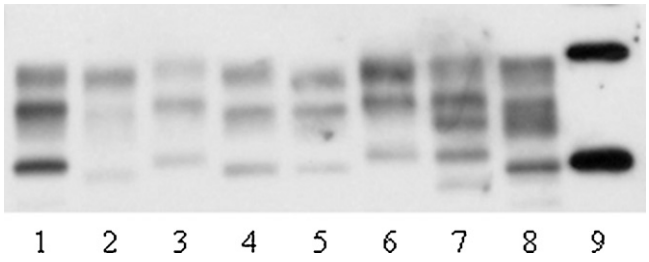

Fig. 7. Molecular patterns of TSE brains obtained on TeSeE™ Western blot assay. Brain homogenates from 6 TSE patients were diluted in Laemmli solution to give comparative PrP^{res} signals. Lane 1: sCJD control type-1 PrP^{res} obtained with UC precipitation; lane 2: vCJD type-2b PrP^{res}; lane 3: sCJD type-1 PrP^{res}; lane 4: sCJD type 2 PrP^{res}; lane 5: FFI D178N type 2 PrP^{res}; lane 6: gCJD E200K type 1 PrP^{res}; lane 7: iCJD GH type-1 PrP^{res}; lane 8: sCJD control type-2 PrP^{res} obtained with UC precipitation. Lane 9: molecular mass marker. The bands correspond to the molecular mass markers of 30 kDa and 20 kDa, respectively, from up to down (sCJD: sporadic Creutzfeldt–Jakob disease; vCJD: variant Creutzfeldt–Jakob disease; FFI: fatal familial insomnia; gCJD: genetic Creutzfeldt–Jakob disease; iCJD: iatrogenic Creutzfeldt–Jakob disease; GH: growth hormone; UC-WB: ultracentrifugation Western blot).

3.3. Discrimination of PrP^{res} molecular pattern

The TeSeE™ Western blot assay could detect the typical PrP^{res} patterns in sporadic CJD (sCJD) types 1 and 2, which were distinguishable from the patterns of vCJD, genetic E200K and fatal familial insomnia (Fig. 7). PrP^{res} isolated from the various categories of TSE patients (variant, sporadic T1, sporadic T2, genetic E200K and fatal familial insomnia) showed the same glycosylation patterns as identified previously by routine UC-WB. The glycoform ratios, although not measured strictly, were similar to those obtained by the routine method.

PrP^{res} was identified clearly with a typical molecular pattern in all vCJD spleen and tonsil samples, whereas no signal was found in patients without CJD (Fig. 4).

3.4. PrPres heterogeneity in tissue

In the prospective study, several areas of each brain were tested by the TeSeE™ CJD ELISA and TeSeE™ Western blot assay. The resulting optical densities showed heterogeneity in the PrP^{res} distribution of TSE patients according to brain area (Fig. 8A) and TSE etiology (Fig. 8B), with a ratio of 50% between two areas of a given brain. TeSeE™ Western blot results presented in Fig. 6 for patient A also showed such heterogeneity: PrP^{res} molecular pattern intensity decreased from striatum (lane 3) to frontal cortex (lane 4) and cerebellum (lane 2). Optical densities obtained for 1/150 brain homogenates from the same sCJD patient on TeSeE™ CJD ELISA showed an identical trend, with 2.620 OD for the striatum, 2.448 OD for the frontal cortex and 2.057 OD for the cerebellum. In addition, the frontal cortex, occipital cortex and striatum from the FFI patient gave low but positive OD values (respectively 0.184, 0.150 and 0.164), whereas the thalamus was positive more clearly (0.456 OD; all marked with an asterisk in Fig. 8A). Finally, as in the brain, variability was found in the ELISA OD values for different areas of the spleen (Fig. 2), reaching 75% for spleen C.

4. Discussion

TeSeE™ Western blot and TeSeE™ CJD ELISA permitted clear discrimination of the 54 TSE patients and the 51 without TSE, with no equivocal results. These data, obtained both on a well characterised panel of brain tissue examined retrospectively and on a prospective blind patient cohort, showed that the full process used for animal TSE was transferred successfully to human application. The monoclonal antibodies used in these assays were selected

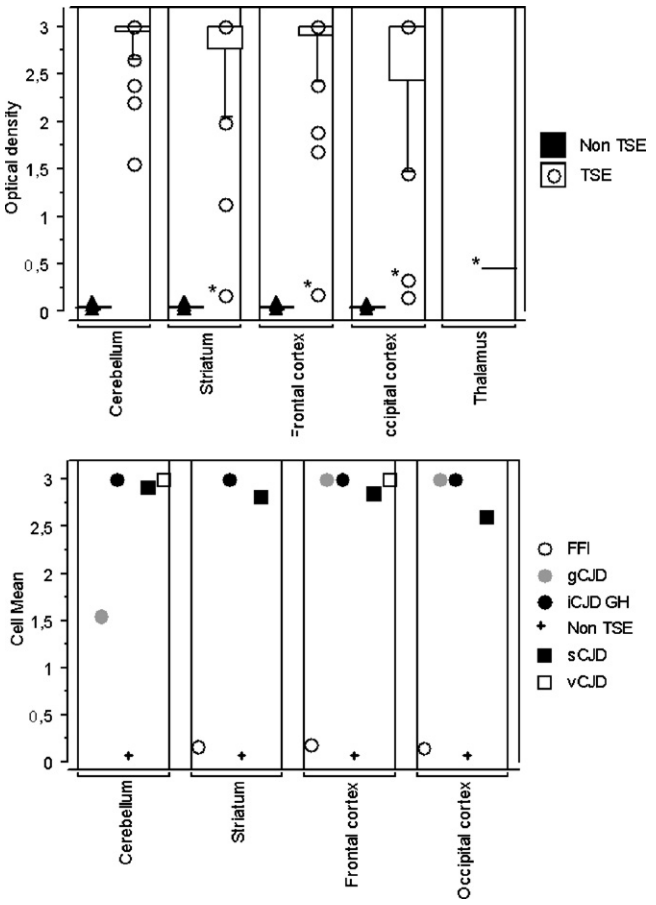

Fig. 8. (A) Box plots of TeSeE™ CJD ELISA: OD distribution in TSE and non-TSE populations in the prospective study. Cerebellum: non-TSE ($n = 31$), TSE ($n = 36$); striatum: non-TSE ($n = 22$), TSE ($n = 26$); frontal cortex: non-TSE ($n = 37$), TSE ($n = 38$); occipital cortex: non-TSE ($n = 20$), TSE ($n = 27$); thalamus: TSE ($n = 1$). (B) Mean cell plots of TeSeE™ CJD ELISA: mean OD in the different TSE etiologies and non-TSE population in the prospective study. Fatal familial insomnia (FFI) is indicated by an asterisk (*). Cerebellum: growth hormone CJD (iCJD GH) ($n = 2$), genetic TSE (gCJD) ($n = 1$), non-TSE ($n = 31$), sporadic CJD(sCJD) ($n = 32$), variant CJD (vCJD) ($n = 1$); striatum: FFI ($n = 1$), iCJD GH ($n = 2$), non-TSE ($n = 22$), sCJD ($n = 23$); frontal cortex: FFI ($n = 1$), iCJD GH ($n = 2$), gCJD ($n = 1$), non-TSE ($n = 37$), sCJD ($n = 33$), vCJD ($n = 1$); occipital cortex: FFI ($n = 1$), iCJD GH ($n = 1$), gCJD ($n = 1$), non-TSE ($n = 20$), sCJD ($n = 24$).

for their sensitive detection of the PK-treated and denatured PrP. The capture antibody 3B5 recognises the octarepeat region of PrP (Krasemann et al., 1996, 1999), while the tracer antibody 12F10 binds to the core part of the protein (Feraudet et al., 2005). PK digestion is carried out in a controlled concentration and a controlled medium (mixture of detergent and chaotropic agents) to preserve the N-terminal epitope. This octarepeat region is present in human PrP and PrP in various animal species, and probably explains in part the successful interspecies transfer found with both tests.

TeSeE™ CJD ELISA detected efficiently PrP^{res} in the brain tissue of all CJD and fatal familial insomnia patients. In this assay, however, a very large and viscous pellet was observed in 10 of the 77 cerebellum homogenates at the end of purification, leading to very heterogeneous samples in which the pellet was not dissolved completely. Consequently, these samples were not tested. This was observed in both TSE-negative (6 cases) and TSE-positive (4 cases) samples, but exclusively in the cerebellum. Thus 4% of samples (10 out of 238 homogenates) were not examined on ELISA. In these 10 patients, the OD for the other brain homogenate areas were available and showed clearly positive or negative OD values, permitting unequivocal diagnosis in these patients. The case of fatal familial insomnia should be noted, because of the weak OD values

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obtained in the various homogenates tested. The cerebellum was not testable, and the frontal cortex, occipital cortex and striatum gave low ODs under 0.2 units absorbance, but strongly positive with respect to the cut-off value determined on non-TSE homogenates (0.081 units absorbance). In the fatal familial insomnia patient, however, absorbance in the thalamus area was clearly higher than the cut-off value (0.456 OD). In the prospective study, the test was slightly positive in one occipital cortex homogenate, whereas the other brain-area homogenates from the same patient were strongly negative; this occipital cortex homogenate was identified correctly as negative on retest.

For tonsil examination, the TeSeE^{TM} CJD ELISA gave good results in the two raw samples tested: OD was 0.151 for the 2-fold diluted sample and 2.034 for the neat sample, and classified as clearly positive, whereas non-vCJD tonsil results were below the cut-off value defined on brain tissue. As in brain, ELISA OD values varied between different areas of the spleen, by as much as a factor of 3. Results on at least two or three spatially separate neat and diluted homogenates were necessary to validate diagnosis with 100% sensitivity and specificity. The heterogeneity of PrP^{res} rates observed in the present ELISA results in different brain areas was well described in the brain (Parchi et al., 2000; Schoch et al., 2006) and the spleen (Peden et al., 2010) and probably also exists in the tonsil. Consequently, it is absolutely necessary to test at least two different areas in the brain and spleen for the post-mortem diagnosis. In the tonsil, all available tissue should be tested for the pre-mortem diagnosis.

The recommended quantity in the extraction kit is respectively 350 mg and 200 mg for brain and lymphoid tissue. However, while not an essential issue for brain examination, this quantity could be difficult to obtain from peripheral tissue such as the tonsil. Some experiments were carried out with small quantities of tissue (data not shown). In order to conserve the 20% tissue homogenate, a proportional decrease in brain material (starting with only 40 mg of tissue) and reagent volume (200 μL 5% glucose) was tested successfully, with the same sensitivity as with 350 mg of tissue. The second possibility examined was to decrease the initial quantity of tissue in order to prepare a more concentrated homogenate: 12.5% homogenate in glucose from only 25 mg of tissue could also offer an alternative. In that case, the volumes of the buffers added successively according to the initial ratio must be adapted correctly. Based on these results, both these tests could enable testing of tiny samples from biopsies that do not show the relevant histopathological changes.

In addition, a dilution study with the NIBSC samples showed that the sensitivity of the two tests enabled detection of weakly CJD-positive brain tissue. For the two NIBSC vCJD samples, TeSeE^{TM} ELISA was able, at the tested dilution, to detect up to 190 nL of sample (corresponding to 19 μg of TSE brain tissue equivalent), whereas TeSeE^{TM} Western blot was able to detect the typical molecular pattern in the equivalent of 80 nL (8 μg). Diluted in 350 mg CJD-negative brain or in 200 mg negative tonsil, 19 μg or 3 μg of CJD-positive brain tissue were respectively enough for TeSeE^{TM} ELISA and TeSeE^{TM} Western blot. Increasing exposure time with ECL+ led to detection of an equivalent of 30 nL (3 μg) of 10% vCJD brain in tonsil tissue homogenate. These data were similar to the reported immunoassay data, using differential PrP extraction in guanidine hydrochloride, quantified by DELFIA technology, which detected an equivalent of 100 nL 10% vCJD brain (NHBYO/0003) spiked in tonsil homogenate (Dabaghian et al., 2006).

The TeSeE^{TM} CJD Western blot test detected successfully all positive brain, spleen and tonsil homogenates, with 100% sensitivity and specificity.

To have a complete evaluation of the TeSeE^{TM} Western blot assay, its capacity for molecular typing of PrP^{res} was tested (Parchi et al., 1999). A varied panel of PrP^{res} molecular patterns from different causes identified previously by routine UC-WB on brain homogenates was compared using the Bio-Rad assay. The TeSeE^{TM} Western blot assay distinguished correctly the different patterns of type-1 and type-2 PrP^{res} and enabled recognition of the different glycoforms of human brain PrP^{res} without significant shift. The vCJD molecular PrP^{res} pattern was discriminated from those found in sporadic or genetic CJD. The exact correlation between the two techniques indicated that this method was compliant for molecular typing of PrP^{res} in TSE. In a few cases, additional bands near 15 or 17 kDa were seen in some positive brain areas by the Bio-Rad assay, as described by Notari et al. (2008) for Western blot using anti-COOH-PrP moiety antibodies.

According to the manufacturer's recommendations, the ECL Western blot detection reagents were used as substrate for the TeSeE^{TM} CJD Western blot test, and results were visualised as necessary by exposure to ECL Hyperfilms for 15 min. However, due to a lack of sensitivity, it was necessary to change the detection reagent and/or expand the exposure time for the spleen and tonsil (Figs. 4 and 5). These modifications sometimes led to the appearance of a weak band above 30 kDa, considered as an artefact (lane 2 Fig. 4, and lane 7 Fig. 5). Nevertheless, even when present, this slight signal was not a source of diagnostic error, as the complete three-band PrP^{res} pattern was not identified. Although this assay should be optimised for tonsil examination to avoid this artefact, these preliminary results allow confidence for the detection of PrP^{res} on raw pre-mortem tonsils from vCJD.

Finally, the determination of the ELISA OD value guides positive sample dilution for WB analysis. Excessively concentrated load in one lane on the gel prevents interpretation of results in neighbouring lanes. Taking account of the ELISA results enables immediately interpretable confirmation of diagnosis on Western blot. With TeSeE^{TM} CJD ELISA, reliable pre-mortem diagnosis could be delivered to clinicians within 4 h, particularly in case of contaminated device isolation. In a second step, the TeSeE^{TM} Western blot assay protocol enables the antibody recognition of the different glycoforms of human brain PrP^{res} to characterise the PrP^{res} pattern. Thus, the vCJD PrP^{res} molecular pattern is discriminated from that found in the sporadic or genetic CJD.

A diagnostic strategy for prion diseases is proposed, using these robust commercial kits. At least two different brain areas (frontal and occipital cortex, striatum or cerebellum) of the suspected CJD patient are to be collected. Each brain area homogenate is prepared from 350 mg of tissue and divided into three aliquots: one of 250 μL for the TeSeE^{TM} CJD ELISA and another of 500 μL for the TeSeE^{TM} Western blot, with the rest as backup. These aliquots are frozen at −80 °C. The TeSeE^{TM} CJD ELISA is carried out with neat and 1/150 R6 diluted homogenate. After 4 h, the corresponding OD values are obtained and the PrP^{res} status is available on a first screening using ELISA. In a second step, if the OD value is below cut-off, the second aliquot is loaded neat for the TeSeE^{TM} Western blot assay; if the OD value is above cut-off, TeSeE^{TM} Western blot assay is carried out with neat and diluted homogenate. The dilution factor is determined so as to obtain a final OD value near 0.015. About 7 h are necessary to confirm the diagnosis of TSE, determine the PrP^{res} molecular pattern, and discriminate sporadic or genetic from variant CJD. In short, with this proposal, 4 h are needed for diagnosis and two days for biochemical confirmation and characterisation of the prion disease.

5. Conclusion

These commercial kits, supplied initially for animal TSE diseases, were validated successfully for detection of PrP^{res} in human brain for diagnostic purposes. In lymphoid tissue, PrP^{res} was detected in the few samples available, and completed the results reported by Clewley et al. (2009) in tonsil study.

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The feasibility study data demonstrated that the TeSeE™ CJD ELISA and TeSeE™ Western blot kits are suitable for detection of PrP^{res} in human brain and lymphoid tissue. They seem to be a good alternative to the time-consuming method using ultracentrifugation. Furthermore, the complete study carried out on a larger cohort of patients confirmed these results, particularly for tonsil, which is unavailable from NIBSC. Using validated commercial tests is a guarantee for biologists in terms of robustness and reproducibility for routine diagnosis purposes and could be helpful in any diagnosis laboratory concerned by Quality Assurance Policy and Accreditation regulation. The TeSeE™ Western blot kit can be used efficiently for confirmation of any TSE-suspected sample with 100% sensitivity and specificity and for typing of TSE strains.

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