Rapid diagnosis of human prion disease using streptomycin with tonsil and brain tissues

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The use of streptomycin in the pathological prion protein (PrP^{sc}) detection procedures represents a new and attractive way for diagnostic purpose. With this agent, western blot readily detected PrP^{sc} in 263K scrapie hamster and C57Bl/6 wild-type mice challenged with C506M3 scrapie strain. Our aim was to evaluate this new diagnosis procedure in the field of human transmissible spongiform encephalopathies (TSEs). First, we had confirmed the ability of streptomycin to precipitate PrP^{res} from human brain of Creutzfeldt–Jakob disease (CJD) patient. Second, we compared the detection of PrP^{res} with streptomycin against three other protocols using other precipitations. Then we assessed PrP^{res} detection with streptomycin in 98 brain tissue samples from various aetiologies of human TSEs and 52 brain samples from other dementia. Finally, we applied this protocol for tonsils examination of five patients suspected of variant CJD (v-CJD). Sensitivity and specificity obtained with the streptomycin protocol were both 100% on brain tissue. For tonsil tissues, PrP^{res} was clearly identified in the two post-mortem confirmed v-CJD cases, whereas no characteristic three-band pattern was seen in the three confirmed non-v-CJD samples. In this study, streptomycin demonstrated its efficiency to detect PrP^{res} both in the central nervous system and in the lymphoid tissue without practical difficulty and with rapid preparation. Because of its ability to act as a good agent for PrP^{sc} examination in different tissues, recovery of PrP^{sc} in biological fluids using streptomycin should open further perspectives of applications in CJD diagnostics. Streptomycin effects *in vivo* might thus also be questioned.

The normal or native prion protein, designated as PrP^{c} for the cellular prion protein, is a widely occurring protein, the sequence of which is well conserved in mammals.[1] Conformational changes in PrP^{c} lead to propagation of the pathogenic prion protein 'PrP^{sc}' that is proteinase K (PK) resistant, at least for its core fragment, which is referred as 'resistant PrP' (PrP^{res}). The PrP^{sc} accumulation in animals is associated with a wide range of diseases classified as 'transmissible spongiform encephalopathies' (TSEs). TSEs cover a series of transmissible neurodegenerative diseases, including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Kuru and Creutzfeldt–Jakob disease (CJD) in humans.[2] As represented by Gerstmann–Straüssler–Scheinker disease and fatal familial insomnia (FFI) in humans, TSE forms may also have genetic origins linked to mutations in the PrP gene that affect thermodynamics of the encoded protein in a spontaneous way favouring beta-sheet transconformation of PrP^{c} to PrP^{sc}. Consequently, a significant probability to evolve towards pathogenic propagation during the lifetime exists in individuals affected by such genetic mutations in their inherited genotype.[3,4] Coincidently with the outbreak of BSE epidemics and the confirmed transmission by food products, a new form of human CJD appeared with original histopathological and biochemical characteristics of PrP^{sc}, which was named 'new variant CJD' (v-CJD).[5,6] It is now admitted that v-CJD originates from the bovine BSE epidemics by passage to humans through alimentation.[7,8]

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The slow development and appearance of symptoms in cattle infected with BSE, with average incubation periods over 4–6 years, have greatly hindered the development of epidemic models as well as the reality of a passage to humans, itself hindered by an even longer period of incubation, which may last for decades.[9] Indeed, the antigenic PrP^{sc} load is generally thought to increase progressively after infection, in lymphoid and peripheral nervous tissue and to reach the brain where its aggregation determines the fatal neuropathological issue of TSEs.[10] Thus, apart from the brain in which PrP^{sc} accumulation is a dead end, PrP^{sc} concentration is expected to be low in other tissues or in biological samples such as blood.[11]

Today, v-CJD epidemics in humans have only clinically emerged in most susceptible individuals, ie with homozygotic methionine genotype in PrP 129 (MET-MET) codon—— known to be associated with short incubation period—and with a window period of alimentary contamination during childhood, when factors such as intestinal barrier functionality are critical.[12] Recently, a novel PrP^{sc} type in a patient exhibiting prion protein gene 129VV polymorphism was reported as atypical because of certain clinical and molecular PrP^{sc} features overlapping with those of v-CJD.[13] Nonetheless, recent studies providing evidence of 'asymptomatic infected carriers' in the UK population, as well as the fourth case of 'highly probable' transfusional cases already reported today are in favour of a possible 'underground propagation' of PrP^{sc} within the human population.[14–18] Though its reality may be considered as evidenced, its extent and its evolution in the exposed populations, within such long delays of asymptomatic incubation and with possible lifelong asymptomatic carrier cases, are still very difficult to estimate.

Therefore, diagnostic tests for the detection of PrP^{sc} infection at onset are required for an efficient control of this disease. In a research strategy for the definition of test principles suitable for the early detection of infection by PrP^{sc} in humans, we have been interested in screening molecules for their ability to capture, concentrate and/or precipitate PrP^{sc}.

After having screened numerous molecules and devices for such properties, one of the selected molecules, streptomycin, is a well-known antibiotic and revealed capable of aggregating PrP^{sc} molecules from different tissue origins with different stages of aggregation.[19] Streptomycin is now used to increase the sensitivity of PrP^{sc} immunodetection using its properties to aggregate and flocculate PrP^{sc} molecules in different tissues of animals challenged for experimental TSE.[20] Moreover, successful precipitation of human brain PrP^{sc} with this agent was reported in feasibility studies compared with animal samples.[19,20] These properties were also used in immunohistochemistry, giving specific and sensitive immonoreactive PrP^{sc} deposits in both natural and experimental scrapie and BSE diagnosis.[21,22]

To ascertain the reliability and specificity of sample treatment with streptomycin for developing diagnostic detection of PrP^{sc} in humans, we have designed a study using streptomycin followed by a reference western blot (WB) immunodetection on well-characterized reference human brain material from various forms of TSE in parallel with equivalent brain material from confirmed non-TSE patients. The protocol was also applied for tonsil tissue from v-CJD patients, in parallel with equivalent material from non-TSE patients. We thus present here the results obtained at first on a cohort of 150 patients from which necropsic human brain tissue was expertised for final TSE diagnosis confirmation by immunohistological and biochemical procedures. They represent 98 TSE cases and 52 non-TSE controls. Second, we present the results obtained on a small cohort of five patients from whom tonsil tissue was assessed for v-CJD diagnostic; two of them were further confirmed as v-CJD after post-mortem brain examination.

MATERIALS AND METHODS
Patient's Cohort
For brain tissue, samples were obtained after necropsy of 150 patients suspected of CJD in anatomopathology laboratories of Lyon, Bordeaux and Marseille. TSE disease was confirmed in 98 patients (neuropathological lesions, immunohistochemistry and immunodetection of PrP^{res} by WB).[23] For positive TSE patients, prion protein (PRNP) gene mutation analysis,[24] codon 129 polymorphism of the PRNP gene,[25] PrP^{res} molecular typing[26] and aetiology are reported in Table 1. Prion disease was rejected in the remaining 52 patients. The final diagnosis of non-TSE patients was further completed: 19 Alzheimer's disease, 4 Lewy body dementia, 4 Parkinson's disease, 2 fronto-temporal dementia and 23 others types of pathologies. For tonsil tissue, samples were obtained after tonsillectomy for diagnostic purpose of suspected v-CJD in five patients. Two patients were classified v-CJD after confirmed diagnostics carried out on brain necropsic tissues, whereas the three others were confirmed negatives for v-CJD diagnosis.

Common Pretreatment of Samples Used in Comparative Study
To compare WB protocols on brain tissue, only available aliquot samples stored at −80°C from the same brain area (prefrontal cortex) were used. In first series, 500 mg of prefrontal cortex was homogenized with distilled water at 20%. Homogenates were forced through a 0.5 mm diameter needle and divided into four aliquots for the different concentration protocols tested. In second series, with the same protocol, we prepared another homogenate from 20 mg of prefrontal cortex and divided it into four aliquots. The precipitation of PrP^{sc} was thus compared with different techniques using a small amount (5 mg) of brain tissue.

Streptomycin Protocol (Streptomycin)
Analytical comparison of four protocols with determination of dilution limit was performed on 25 μl of the 20%

Table 1 Diagnostic classification of studied TSE patients

No. of cases	Aetiology	PrP^{res} molecular typing/number of samples/PRNP mutation		Prion protein codon 129 polymorphism
80	Sporadic	Type 1; $n = 61$	Type 1	MM; $n = 50$;
				MV; $n = 8$
			Type 1 majority+type 2	MM; $n = 3$
		Type 2a; $n = 19$	Type 2	MM; $n = 3$;
				MV; $n = 5$;
				VV; $n = 9$
			Type 2 majority+type 1	MM; $n = 1$;
				VV; $n = 1$
14	Genetic	Type 1; $n = 9$	E200K	MM; $n = 6$;
				VV; $n = 3$
		Type 2a; $n = 2$	V203I	MM; $n = 2$
		Type 1; $n = 1$	D178N CJD	VV; $n = 1$
		Type 2; $n = 2$	D178N FFI	MM; $n = 2$
2	Iatrogenic: growth hormone	Type 1; $n = 2$		MM; $n = 1$,
				MV; $n = 1$
2	Variant	Type 2b; $n = 2$		MM; $n = 2$

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

Necropsic brain tissues have been collected and treated according to the French ethical and technical legislation. Tonsil samples have been collected for diagnostic purpose of variant CJD.

homogenate (corresponding to 5 mg equivalent brain tissue) diluted twofold in phosphate-buffered saline (PBS) $2 \times$ (pH = 7.2, DOC 1%, NP-40 1%).

For the cohort examination, 5 mg of prefrontal cortex was directly homogenized in PBS (pH = 7.2) DOC 0.5%, NP-40 0.5% representing a final 10% brain homogenate. For tonsil tissues, 10% homogenates were simply made in 5% glucose. Tissues were all treated with PK (80 μg/ml) for 30 min at 37°C and streptomycin (200 mg/ml) was added for 1 h at 37°C as previously described.[19] Thus, after having made 10% homogenates from 5 mg of TSE brain, we used a 0.14 M final concentration of streptomycin in PK incubation medium. Samples were then submitted to centrifugation for 15 min at 17 000 g. A second centrifugation was made when a slightly milky zone was observed above the pellet. Pellets were re-suspended in 4% SDS denaturing buffer (Tris HCl pH = 6.8 125 mM, glycerol 20%) and boiled for 10 min at 100°C. Then, they were further processed as indicated below for WB analysis.

Ultracentrifugation Protocol
For the analytical comparison of the four protocols, 500 or 25 μl of the 20% homogenate (corresponding to 100 or 5 mg equivalent brain tissue respectively) was diluted twofold in 10% glucose.

In our laboratory, this protocol is currently performed since 1998 with 100 mg of prefrontal cortex as previously described[27] with slight modifications; prior to ultra-centrifugation in Sarkosyl, homogenates were digested by proteinase K (25 μg/ml) for 1 h at 37°C before stopping digestion by PMSF 1 mM.

Sodium Phosphotungstic Acid Protocol
For the analytical comparison of the four protocols, 250 or 25 μl of the 20% homogenate (corresponding to 50 or 5 mg equivalent brain tissue, respectively) was diluted twofold in PBS buffer $2 \times$ without Ca^{++} and Mg^{++} (pH = 7.2). The protocol was strictly performed as previously described with 500 or 50 μl of 10% homogenate (50 or 5 mg) of prefrontal cortex.[28]

Centrifugation Step Concentration Protocol (LC)
For the analytical comparison of the four protocols, 40 or 25 μl of the 20% homogenate (corresponding to 8 or 5 mg equivalent brain tissue, respectively) was diluted twofold in Tris-buffered Saline $2 \times$ (pH = 8). The protocol corresponding to the 'Sample preparation II' described by Lee *et al.*[29] was performed as previously described with 8 or 5 mg of prefrontal cortex.

WB Immunodetection
Each sample was analysed on 12% Nu-PAGE gels. Gels were then electroblotted on to PVDF membranes and blocked with 5% (W/V) milk powder in PBS for 1 h.
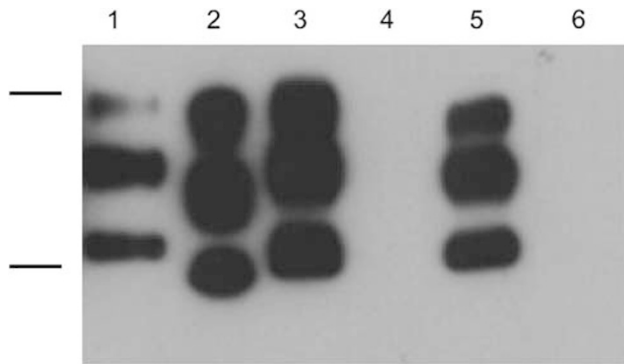
Figure 1 Analysis of supernatants after streptomycin precipitation on 5 mg CJD brain. Immunoblot analysis of prefrontal cortex homogenates obtained from different suspected CJD patients is shown. Black lines correspond to molecular mass marker of 33 and 20 kDa, up to down, respectively. PrP^res is immunodetected by 3F4 Ab. Lanes 1 and 2: PrP^res types 1 and 2, respectively, extracted using ultracentrifugation protocol. Lanes 3 and 4, and 5 and 6: PrP^res type 1 from two CJD patients, extracted using streptomycin protocol, respectively pellets (lanes 3–5) and supernatants (lanes 4–6).
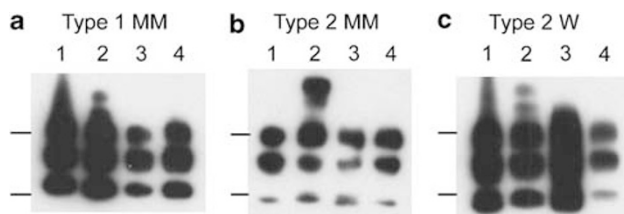

Figure 3 Comparison of PrP^res signals after performing the four protocols with initial 5 mg of brain tissue. Immunoblot analysis of prefrontal cortex homogenates obtained from different suspected CJD patients is shown. Black lines correspond to molecular mass markers of 33 and 20 kDa, up to down, respectively. Here, 5 mg of brain tissue was performed for each protocol and then 1 mg equivalent brain was load in each well. PrP^res is immunodetected by 3F4 Ab. (**a**) s-CJD Met/Met type 1 PrP^res; (**b**) FFI Met/Met type 2 PrP^res and (**c**) s-CJD Val/Val type 2 PrP^res. Lanes 1: samples processed by the ultracentrifugation protocol (UC). Lanes 2: samples processed by the centrifugation step concentration protocol (LC). Lanes 3: samples processed by the sodium phosphotungstic acid protocol (NaPTa). Lanes 4: samples processed by the streptomycin protocol.

Figure 2 (**a**–**c**) Comparison of PrP^res signals after different protocols of precipitation with the recommended initial amount of tissues. Immunoblot analysis of prefrontal cortex homogenates obtained from different suspected TSE patients is shown. Black lines correspond to molecular mass marker of 33 and 20 kDa, up to down, respectively. Here, 1 mg equivalent brain was load in each well. PrP^res is immunodetected by 3F4 Ab. (**a**) s-CJD type 1 PrP^res; (**b**) FFI type 2 MM PrP^res and (**c**) s-CJD type 2 VV PrP^res. Lanes 1: samples processed by the ultracentrifugation protocol (UC). Lanes 2: samples processed by the centrifugation step concentration protocol (LC). Lanes 3: samples processed by the sodium phosphotungstic acid protocol (NaPTa). Lanes 4: samples processed by the streptomycin protocol.

Immunodetection of PrP^res was observed using 1/10 000 3F4 purified anti-PrP monoclonal antibody (Proteogenics, USA) for 1 h at room temperature and revealed by chemiluminescence substrate West Dura (Pierce, USA).

RESULTS
Analytical Features

As shown in Figure 1, PrP^res signal is totally collected in the pellet as no immunodetection was evidenced in the supernatant. Thus, this concentration corresponding to 0.14 M of streptomycin in PK digestion medium was adopted for following validation in human TSE brain and tonsil samples.

We then compared the ability of streptomycin concentration *vs* our usual technique and *vs* other established sensitive protocols. To observe signals on the same membrane after
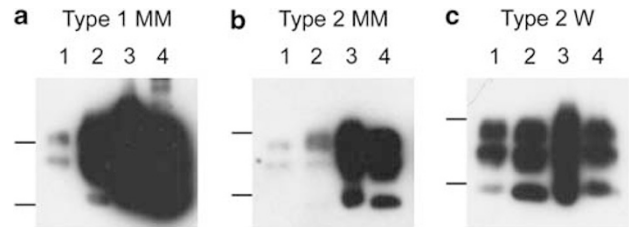
immunodetection, we loaded in each well 1 mg equivalent brain tissue obtained for each of the four different methods. As shown in Figure 2a–c, the detected PrP^res signals indicated that streptomycin was a pertinent agent of concentration of PrP^res in the three different types of CJD samples. Indeed for the FFI Met/Met type 2 sample, signals with the four techniques were very similar, whereas for CJD Met/Met type 1 PrP^res, signals appeared to be slightly more important with ultracentrifugation protocol (UC) and LC protocols than with streptomycin and NaPTa protocols. Finally, for CJD Val/Val type 2, NaPTa and UC protocols gave better PrP^res signals than the two others.

In the second series, we compared the analytical sensitivity and the ability of each protocol to recover PrP^sc from only 5 mg of brain tissue. As shown in Figure 3, NaPTa and streptomycin protocols were the most efficient procedures for good recovery of PrP^res in s-CJD Met/Met 1 (Figure 3a) and genetic CJD Met/Met 2 (Figure 3b), whereas no significant difference was seen for s-CJD Val/Val 2 samples (Figure 3c). For s-CJD Met/Met 1, we observed that UC protocol (Figure 3a, lane 1) was less sensitive, leading only very faint PrP^res signal on overexposed WB. Dilution studies on s-CJD Met/Met 1 and Val/Val 2 permitted to confirm that NaPTa and streptomycin were the most sensitive protocols (data not shown). For FFI Met/Met 2 samples, serial dilutions of 5-, 10- and 20-fold were performed, corresponding to 0.2, 0.10 and 0.05 mg of tissue loaded on gel. As shown in Figure 4, we also observed that NaPTa and streptomycin were the most sensitive protocols, whereas the two others were not able to detect PrP^res in these diluted samples. We could also confirm that, in the cases of s-CJD Met/Met 1, s-CJD Val/Val 2 and FFI Met/Met 2, the dilution limit differed as expected from known variation of PrP^sc concentration in corresponding samples.
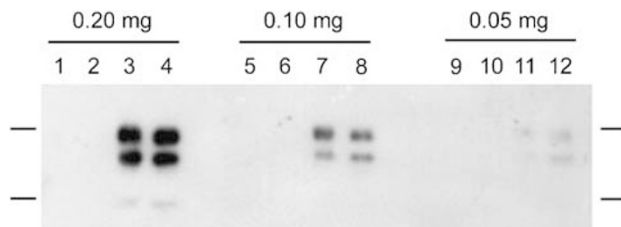
Figure 4 Detection of PrP^{res} after dilution of type 2 Met/Met FFI sample. Immunoblot analysis of prefrontal cortex homogenates obtained from one genetic (TSE) patient with FFI is shown. The sample of FFI type 2 Met/Met PrP^{res} was serially diluted to obtain 0.20 mg (lanes 1–4); 0.10 mg (lanes 5–8) and 0.05 mg (lanes 9–12) of brain tissue. Lanes 1, 5 and 9: samples processed by the ultracentrifugation protocol (UC). Lanes 2, 6 and 10: samples processed by the centrifugation step concentration protocol (LC). Lanes 3, 7 and 11: samples processed by the sodium phosphotungstic acid protocol (NaPTa). Lanes 4, 8 and 12: samples processed by the streptomycin protocol.

Figure 5 Analysis of brain PrP^{res} of suspected CJD patients using streptomycin precipitation (representative selection). Immunoblot analysis of prefrontal cortex homogenates obtained from different suspected CJD patients is shown. Black lines correspond to molecular mass markers of 33 and 20 kDa, up to down, respectively. Lane 1: control CJD PrP^{res} type 2 (ultracentrifugation protocol); Lanes 2–7: non-CJD patients; Lanes 8–11: CJD patients.

Cerebral PrP^{res} Detection Using Streptomycin Precipitation

All brain specimens from the 98 confirmed TSE patients showed PrP^{res}-positive signal on WB by immunodetection with 3F4 monoclonal antibody after streptomycin precipitation. As represented in Figure 5, the three-band PrP^{res} pattern is clearly identified for these samples. When the signal was saturated, a smear all along the lane was found. For the specimen of the 52 non-TSE patients (examples in Figure 5), no signal was detected indicating that (i) the digestion of PrP^c by PK was complete, and (ii) streptomycin addition did not interfere with immunodetection.

Most importantly, TSE and non-TSE patients were correctly classified by the assay leading to specificity and sensitivity at 100%.

Cerebral PrP^{res} Molecular Typing Using Streptomycin Precipitation

The evaluation of streptomycin influence on the PrP^{res} molecular typing was further analysed. Using streptomycin precipitation, we were able to detect the two major WB bands with distinct apparent molecular weights of unglycosylated isoform that characterized types 1 and 2 PrP^{res}. So types 1 and 2 PrP^{res} were clearly discriminated. Although we did not perform exact determination of the ratio diglycosylated/ monoglycosylated bands, we nevertheless examine PrP^{res} molecular pattern obtained on different brain area and brain extracts in different experiments with streptomycin precipitation. The characteristic v-CJD glycotype in type 2b PrP^{res} seemed to be conserved (Figure 6, lane 6) and was also easily discriminated from those of sporadic type 2a (Figure 6, lane 4) and moreover, from type 1 PrP^{res} (Figure 6, lanes 3 and 5). Concerning type 1 PrP^{res}, characteristic molecular patterns for sporadic (Figure 6, lane 3) and E200K PRNP mutation (Figure 6, lane 5) are clearly distinguishable too.
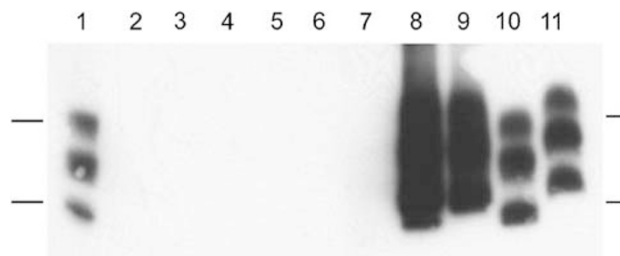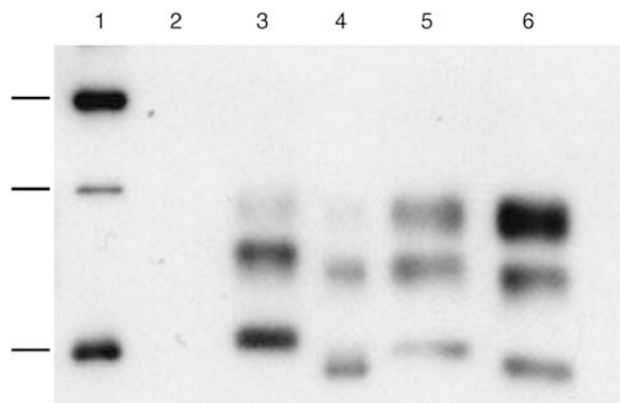

Figure 6 Distinction of PrP^{res} types 1 and 2 using streptomycin protocol. Immunoblot analysis of prefrontal cortex homogenates obtained from different CJD patients is shown. Black lines correspond to molecular mass markers of 40, 30 and 20 kDa, up to down, respectively. PrP^{res} is immunodetected by 3F4 Ab. Lane 1: molecular markers, respectively from up to down: 40, 30 and 20 kDa. Lane 2: non-CJD control sample. Lane 3: type 1 PrP^{res} (sporadic CJD patient); lane 4: type 2a PrP^{res} (sporadic CJD patient); lane 5: type 1 PrP^{res} (genetic CJD: E200K PRNP mutation); lane 6: type 2b PrP^{res} (v-CJD patient).

Tonsil Examination for PrP^{res} Detection with Streptomycin Protocol

The type 2b PrP^{res} was clearly identified in the two tonsils extracted from confirmed v-CJD cases (Figure 7, lanes 4 and 5) with the characteristic three-band pattern, whereas the three non-v-CJD showed a background and nonspecific signal around 30 kDa that did not fit with any characteristic three-band pattern of PrP^{res} (Figure 7, lanes 1–3). In v-CJD tonsils, the relative ratio of glycotype appeared to be consistent with that of corresponding brain extract.

DISCUSSION

The streptomycin precipitation protocol performed in this study is adapted from the one reported for brain PrP^{sc} detection in natural scrapie, natural BSE and experimental
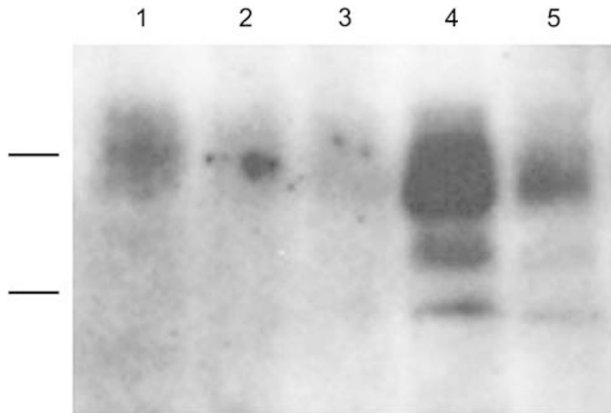
Figure 7 Tonsil analysis for PrP^{res} detection in five suspected v-CJD cases using streptomycin protocol. Immunoblot analysis of tonsil homogenates obtained from five suspected v-CJD patients is shown. Black lines correspond to molecular mass markers of 30 and 20 kDa, up to down, respectively. PrP^{res} is immunodetected by 3F4 Ab. Lanes 1–3: non-v-CJD patients' tonsil examination using streptomycin protocol. Lanes 4 and 5: v-CJD patients' tonsil examination using streptomycin protocol.

BSE in mice.[19] Analysis of the supernatants after precipitation from a very low amount of human brain (5 mg) compared with that routinely used for UC (100 mg) shows that all PrP^{res}-specific signal detected can be concentrated in the pellet, without significant residual signal in supernatant. So the full process used in animal TSE was successfully transferred to human application without modification.

Although the main objective was not a complete analytical study, we nonetheless compared this protocol with different reported processes using concentration steps as UC commonly used in our lab for CJD diagnosis,[27] a centrifugation protocol (LC)[29] and NaPTa step precipitation (NaPTa)[28] as previously described by others. In first series, these concentration protocols were tested as reported without modification prior to a unique WB analysis. Here, the amounts of tissue tested were those recommended for streptomycin, LC, NaPTa and UC protocols (5, 8, 50 and 100 mg, respectively). Thus, comparison of an equivalent quantity was achieved in WB analysis by loading 1 mg of brain tissue extract into the gel. In second series, we have compared the ability of each protocol to recover PrP^{res} from a small amount of brain tissue equivalent to that processed in streptomycin protocol (5 mg). This reproduced situations when only small quantities of original sample material are available (ie tonsils).

From the present study, advantages and drawbacks of each protocol can be rapidly listed. Elimination of most of soluble proteins by UC allows loading high amounts of tissue (20–50 mg) without unspecific noise (not shown) but it is (i) time consuming (4 h 35 min), (ii) large serial analysis cannot be performed within 1 day and (iii) recovery of the pellet needs experience with an important impact of manipulators' skill (particularly when initial quantity is below 10 mg of

tissue). Moreover, this technique revealed poor sensitivity for recovering PrP^{res} when performed from only 5 mg of tissue, in particular for s-CJD Met/Met 1 and FFI Met/Met 2. It thus appeared to be extremely dependent upon the initial amount of tissue. With NaPTa, high recovery of PrP^{res} in the pellet *vs* PrP^{c} offers a potential to load high amounts of tissue (25–50 mg) without unspecific noise but requires (i) long sequential steps (3 h 30 min), (ii) time-consuming buffer and stock solution preparation and (iii) frequent need to solubilize a white precipitate as reported.[28] Nonetheless, NaPTa also succeeded in recovering PrP^{res} in the different PrP^{sc} strains tested here from 5 mg of brain tissue. Using the centrifugation method (LC),[29] centrifugation at 20 000 g reduced the preparation time (3 h 05 min) whatever the amount of tissue. However, with the recommended initial amount of 8 mg of tissue, PrP^{res} was clearly recovered, but when decreased to only 5 mg, the sensitivity dropped. For LC, ratio of proteinase K/amount of brain tissue is the highest of the four techniques, still proteinase K concentration needed to be increased to completely digest PrP^{c} for few cases. Finally, streptomycin concentration gave specific results with good sensitivity, with the lowest amount of tissue and the shortest preparation time (2 h 30 min). Only in few cases complete recovery of pellet required a second low-speed and short centrifugation step. In dilution experiments, the most sensitive protocols were found to be NaPTa and streptomycin.

The main objective was thereafter to evaluate the sensitivity and specificity of the streptomycin protocol in a cohort representing the diversity of human TSE types.

The results obtained here with the post-mortem diagnosed cohort of 98 TSE patients and 52 non-TSE patients yielded 100% specificity and 100% sensitivity, showing neither false positive nor false negative. This performance with such specificity provides criteria enabling the use of this new protocol for PrP^{res} concentration. Indeed, no false negative could be admissible in the field of human TSE diagnosis. Actual sensitivity of the protocol also complies with the objective of detecting brain PrP^{res}, for post-mortem diagnostic purposes. Thus, precipitation by streptomycin was found to be helpful in terms of practicability, and could replace a cumbersome UC method with its different drawbacks. This protocol gave similar signal than the two other methods used, ie NaPTa and LC, when the same quantities of brain tissue are loaded in the gel. For post-mortem brain diagnosis, a decrease in the amount of tissue is not a critical step. But for ante-mortem biopsies of tonsils and other tissues as reported for olfactory mucosa and in our previous study on peripheral nerves,[27,30] processing low amounts without a good recovery of PrP^{sc} would be a limitation for certain methods. In the case of tonsil examination in clinically suspected v-CJD cases, our results obtained with streptomycin permitted to clearly detect characteristic PrP^{res} pattern in this lymphoid tissue. Even if a faint signal can be detected around 30 kDa on non-v-CJD tonsil samples, no

characteristic pattern of PrP^{res} can be detected on overexposed WB with these negative samples, whereas it clearly exists for positive samples (data not shown). We have noticed incidentally that freezing–thawing cycles have decreased this nonspecific signal, whereas increasing PK concentration up to twofold had no influence. Some optimization should be performed to improve specificity if single band analysis ever became relevant, but this streptomycin protocol should be sufficient for a global rapid test. These results were correlated with the confirmed diagnosis obtained after brain examination, confirming again a good sensitivity and specificity. These data, as those recently reported in C57Bl/6 mice inoculated i.c. or i.p., provide evidence that PrP^{res} could be detected either in lymphoid organs or in brain with a good sensitivity.[31] Streptomycin seems then to be an efficient tool for peripheral tissue examination in living individuals.

The present protocol permits the recognition of the different glycoforms of human brain PrP^{res} by 3F4 antibody with the expected shift in the molecular weight.[19] This shift does not alter the classical three-band pattern and therefore, this method complies with the molecular PrP^{res} typing field.[23] Finally, v-CJD molecular PrP^{res} pattern is discriminated from those found in sporadic or genetic CJD. Indeed, in our present conditions, we have worked with streptomycin quantity within the range of those shown not to increase molecular weight patterns in precipitated material from PK-digested homogenate.[19] Moreover, in another study,[20] still different patterns were reported using protocols inverting the proteinase K and streptomycin incubation steps when compared with our protocol. They thus yielded patterns diverging from our own results, which we have obtained after setting up conditions most appropriate for our study dedicated to human CJD diagnostics. Thus our analyses had to be comparable in routine conditions to other reference techniques used in the human medical domain.

In conclusion, it is the first report to our knowledge on streptomycin WB for routine diagnosis of human TSEs. Streptomycin seems to act as a good precipitation agent for low amount of tissue containing PrP^{sc} and thus permits quick analyses on small quantities of tissues, with particular interest for biopsies. This protocol allowed us to detect PrP^{res} in small amounts of tonsil tissue for v-CJD patients. For peroneal nerve analysis in s-CJD as already reported,[27,32] it could also be an alternative to UC as only 20% of sensitivity was found. Streptomycin also seems to be a valid alternative protocol to NaPTa when small amounts of tissue are available. Because satisfactory recovery efficiency of PrP^{res} spiked in CSF and urine was recently claimed,[20] it could now be evaluated as a tool for a test detecting PrP^{sc} in human and animal biological fluids. Finally, because streptomycin is a well-known antibiotic available for human therapy, these *in vitro* results could raise questions about its eventual effects *in vivo*.

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