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RESEARCH ARTICLE

Inter-Laboratory Assessment of PrP^{Sc} Typing in Creutzfeldt–Jakob Disease: A Western Blot Study within the NeuroPrion Consortium

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Abstract

Molecular typing is of considerable importance for the surveillance and epidemiology of human transmissible spongiform encephalopathies (TSEs). It relies on the detection of distinct protease-resistant prion protein (PrP^{Sc}) core fragments that differ in molecular mass and/or glycoform ratio. In this collaborative study, we tested the inter-laboratory agreement in TSE molecular typing. Sixteen characterized brain specimens from sporadic TSEs and variant Creutzfeldt-Jakob disease (vCJD) cases were distributed blindly to seven laboratories for molecular characterization by a defined protocol and classification. Agreement between laboratories in the classification of samples was excellent. In particular, there were no differences in the distinction between PrP^{Sc} type 1, type 2A, and type 2B with one exception, which eventually was identified as a case with types 1 and 2 co-occurrence. This shows that the general technique and particular classification system used here are robust and represent a reliable basis for diagnostic and epidemiologic purposes. The subtle further distinction of subtypes among type 1 and type 2 groups requires high-sensitivity gel electrophoresis protocols that are unsuitable for routine diagnostic needs and must be reserved for research investigations. Further research is necessary on the identification and significance of co-occurrence of PrP^{Sc} types 1 and 2 within one brain.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurodegenerative disorders that affect humans and many mammalian species. They include Creutzfeldt-Jakob disease (CJD) and other less common forms in humans, scrapie in sheep and goats, chronic wasting disease in deer and elks, and bovine spongiform encephalopathy (BSE) in cattle.

At the molecular level, TSEs are characterized by the accumulation of a pathological isoform of the cellular prion protein (PrP^{C}), designated as scrapie prion protein (PrP^{Sc}) (30). The infectious agent, the prion, seems to consist largely or exclusively of PrP^{Sc} (30). PrP^{Sc} is formed from PrP^{C} through a posttranslational event associated with an increase in β-sheet secondary structure leading to an increased aggregation of the protein and the acquisition of partial resistance to protease digestion (6, 21). Incubation of a brain homogenate from prion-infected individuals with proteinase K (PK) under conditions leading to a complete degradation of PrP^{C} generates an N-terminal truncated form of PrP^{Sc}, commonly referred to as PrP27-30, which represents an established diagnostic marker for the disease (3, 32).

TSEs are a phenotypically heterogeneous group of disorders comprising a broad spectrum of clinicopathological variants (8, 16). This is related to the existence of different prion strains which can be distinguished by their disease characteristics after transmission to inbred animals (4). Host genetic factors, in particular polymorphisms or mutations in the coding region of the prion protein gene (*PRNP*), may also significantly affect the disease phenotype. Prion strains have been originally isolated by transmission to inbred mouse lines, a very laborious, expensive

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and time-consuming practice. More recently, however, it has been shown that there is a strong correlation between the particular physicochemical properties of PrP^{Sc} and the pathological and clinical disease phenotypes, and that PrP^{Sc} heterogeneity involves both the site of protein cleavage by proteases and the degree of protein glycosylation (13, 17, 22, 23, 25, 27, 7, 34, 9, 10; for reviews of more recent studies, see 28 and 1). Furthermore, increasing evidence from transmission studies indicates that the different PrP^{Sc} "types" showing distinct physicochemical properties may indeed represent genuine biochemical signatures of individual strain–host genotype interactions (2, 24, 33, 15, 18, 14). As the analysis of biochemical properties of PrP^{Sc} is much less time-consuming than bioassays in mice, unraveling the physicochemical properties of PrP^{Sc} associated with each TSE strain or phenotype (ie, PrP^{Sc} "typing") has become of crucial importance for strain typing and molecular classification of TSEs, with wide implications for both disease diagnosis and epidemiologic surveillance. If a solid correlation between PrP^{Sc} profiling on tissue homogenates of the natural host and strain typing in animal bioassays will be established, there will be a significant reduction of the need to perform transmission studies *in vivo*.

Among the classifications of human PrP^{Sc} types that have been proposed to date (22, 25, 35, 38, 12), the original typing scheme by Parchi *et al* (22, 23) remains the most widely used. This classification is based on the distinction of two major human PrP^{Sc} types: type 1, with a relative molecular mass of 21 kDa and the primary cleavage site at residue 82, and type 2, with relative molecular mass of 19 kDa and the primary cleavage site at residue 97 (22, 23, 27). The two PrP^{Sc} types in conjunction with the codon 129 genotype largely correlate with phenotypic variability in human sporadic TSEs and provide a molecular basis for disease classification (ie, MM1, MM2, VV1, etc.) (25, 26). In addition, variant CJD (vCJD), a novel CJD phenotype originating from a bovine prion strain (36, 5), also correlates with the presence of type 2 PrP^{Sc}, but is distinct from that found in sporadic CJD (sCJD) and sporadic fatal insomnia (sFI) as a result of a characteristically high amount of the PrP^{Sc} glycoform with both N-linked glycosylation sites occupied (7, 23).

Two alternative classifications of human PrP^{Sc} types can be found in the literature (7, 35, 38), both of them indicating that the sCJDMM1 subtype according to Parchi *et al* (25) is a heterogeneous group that includes two distinct PrP^{Sc} isoforms and two disease phenotypes. More recently, however, the study of the effects of stringent pH conditions on the PrP^{Sc} core generated by protease digestion PrP^{Sc} using a gel electropheresis technique with increased sensitivity has resolved these problems of diverging nomenclature and molecular classification (19). It seems that significant pre-existing pH variations between CJD brain homogenates in standard buffers, which influence PK activity and, as a consequence, the size of PrP^{Sc} after cleavage, provide a solid technical explanation for the heterogeneity of type 1 gel migration that had been used to identify putative PrP^{Sc} subtypes. Furthermore, this study (19) demonstrates that PrP^{Sc} types 1 and 2 can be further distinguished into molecular subtypes that better fit the current histopathologic classification of human sporadic TSEs into six subtypes.

The critical steps for the full validation of a molecular strain typing approach based on PrP^{Sc} profiling by Western blot include the standardization and harmonization of PrP^{Sc} typing practice among laboratories, particularly those involved in TSE surveillance. To this aim, a collaborative study between seven laboratories involved in TSE surveillance in different European countries was set up to compare the classification assigned and to assess the robustness of the most widely used method of PrP^{Sc} typing. Laboratories were sent samples of brains from cases of CJD and sFI with the detailed protocols to be followed in determining the type present according to the classification proposed by Parchi *et al* (25), as well as the more detailed subtyping recently proposed by Notari *et al* (19).

MATERIALS AND METHODS

Participating laboratories

Seven laboratories participated in the study (see affiliations for the complete list), all of which returned full reports of the results and copies of the immunoblots to allow independent third-party assessment as requested.

Specimens studied

Samples were provided by laboratories 1 (RV8, RV13, RV14, RV16, RV18, RV19, RV20, RV23), 2 (RV6, RV17, RV12), 3 (RV1, RV4), 4 (RV21, RV22) and 5 (RV5). Materials had been characterized in the originating laboratories to ensure that they included all known sporadic TSE subtypes and vCJD [2 sCJDMM1; 2 sCJDMV1; 2 sCJDVV1; 2 sFI, also referred to as sCJDMM2T (T = Thalamic) (25, 26); 2 sCJDMM2C (C = Cortical); 2 sCJDMV2; 2 sCJDVV2 and 2 vCJD were included]. Specimens were sent on dry ice in the form of unhomogenized tissue fragments together with the operative instructions. All samples were sent blinded with a combined letter and numerical code. The primary assessment of the data was made by the participating laboratories. Reassessments were made in laboratories 1 and 2. The protocol to be followed was taken from Notari *et al* (19), with some modifications, and is specified in detail (see further).

Preparation of brain homogenates

Brain tissues (about 50-mg wet tissue each) from the cerebral cortex were homogenized using disposable polypropylene pestles with matched 1.5-mL microcentrifuge tubes. Ten percent brain homogenates (w/v) were prepared on ice in 9 vol of lysis solution with high buffer capacity containing 100 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5% (v/v) Nonidet P 40, 0.5% (w/v) sodium deoxycholate, 100 mmol/L Tris–HCl, pH 6.9. As the pH of Tris buffers fluctuates significantly depending on the buffer temperature, the lysis buffer was titrated to pH 6.9 $\pm$ 0.1 at 37 $\pm$1°C (ie, the temperature at which protease digestion is performed). In case a significant amount of macroscopically visible debris was present, it was recommended to perform a brief microcentrifugation step (1000 $\times$ g for 10 $\pm$ 1 minute), after which the supernatant was saved and the pellet discarded.

PK digestion and electrophoresis

For each sample, 20 µL of 10% brain homogenate was mixed with 2 µL of 100 U/mL PK solution (equivalent to 5 mg PK/mL when

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the specific PK activity, indicated by certificate of analysis obtained from the manufacturer, is 20 U/mg) corresponding to a final concentration of 10 U/mL (equivalent to 0.5 mg PK/mL), and then incubated for 1.0 ± 0.1 h at 37 ± 1°C. The reaction was terminated by the addition of 3 mM (final concentration) phenylmethylsulfonyl fluoride (Sigma, Sigma-Aldrich, St. Louis, MO, USA). Samples were then resuspended in the sample buffer [final concentrations: 3% (w/v) sodium dodecyl sulfate (SDS), 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 2 mmol/L EDTA, 62.5 mmol/L Tris, pH 6.8] and boiled for 8 ± 1 minutes before loading.

For electrophoresis, samples (10 μL each equivalent to 0.25-mg wet tissue) were loaded into the 12 wells of manually casted 13% Tris–glycine gels (1-mm thick) with a separating distance of 6.5 cm. Protein separation was performed in running buffer [25 mmol/L Tris, 190 mmol/L glycine, 0.1% (w/v) SDS] for 20 ± 2 minutes at a constant voltage of 120 V and subsequently at 180 V until the end of the run using a Criterion cell apparatus (Bio-Rad, Hercules, CA, USA). Subsequently, the separated proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, Billerica, MA, USA) for 2.0 ± 0.1 h at 350 mA using prechilled running buffer supplemented with 20% (v/v) methanol in a Criterion blotter. An electrophoretic system of higher resolution as described in Notari *et al* (19) was also used by some laboratories. In particular, for this setting, manually casted 13% Tris–glycine gels (1-mm thick) with a separating distance of 15 cm and the PROTEAN II xi system (Bio-Rad) were used.

Pre-stained low-range sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein standards (Bio-Rad) were used on each gel to allow continuous monitoring of protein separations during electrophoresis and to assess blotting efficiency.

In order to achieve even signal intensities among the samples, it was recommended to adjust the PrP^{Sc} content of those samples displaying (i) a relatively strong signal by diluting them in 1× sample buffer and (ii) a very weak signal by precipitating them with 4 vol of methanol and subsequently to resuspend them in a smaller volume of 1× sample buffer, respectively.

Immunoblotting

After the electrotransfer, PVDF membranes were immediately incubated in blocking buffer (10% (w/v) nonfat dried milk, in Tween-Tris Buffer Saline (T-TBS) buffer [0.1% (v/v) Tween 20, 0.9% (w/v) NaCl, 50 mmol/L Tris–HCl, pH 7.6] for 1.0 ± 0.1 h at 37 ± 1°C under gentle shaking. The blots were washed with T-TBS (a few seconds, four to five times) and then incubated with anti-PrP antibody 3F4 (Signet Labs, Dedham, MA, USA) diluted in antibody dilution buffer [1% (v/v) normal goat serum, 0.05% (w/v) Bovine serum albumine (BSA), 0.01% (w/v) thimerosal] at 1:40 000 overnight at 4 ± 2°C. The blots were washed four times (7 minutes each) with T-TBS, followed by incubation with a sheep antimouse antibody conjugated with horseradish peroxidase (GE Healthcare, Milan, Italy) diluted in antibody dilution buffer at 1:3 000 for 1 ± 0.1 h at room temperature. After four washes with T-TBS (7 minutes each), the immunoreactivity was visualized by enhanced chemiluminescence (GE Healthcare) on Kodak BioMax films (Kodak, Rochester, NY, USA) according to the manufacturer's instructions. Densitometric analysis was performed using the current equipment in use in each of the participating laboratories.

RESULTS

In a first round of Western blots, the samples were identified as type 1 or type 2A or type 2B. In the second round, the type 1 and type 2 (either A or B) samples were each run side by side on two separate gels to confirm the type 1/type 2 classification and to identify more subtle difference in gel migration within each of the two groups of samples (ie, types 1 and 2). Examples of immunoblots, illustrating the basis of the classification methods by Parchi *et al* (22, 23) and Notari *et al* (19), are shown in Figures 1 and 2. In Figure 1, several samples separated in a conventional Tris–glycine gel electrophoresis system are shown. It can be observed that the band corresponding to unglycosylated PrP^{Sc} of samples RV4 and RV19 migrates at the same rate, while the corresponding band of samples RV12, RV13, RV16, RV20 and RV21 migrates faster. Therefore, according to Parchi *et al* (22, 23), the first group (Figure 1A) is classified as type 1, while the second is classified as type 2. In addition, inspection of the upper two bands, representing the di- and the monoglycosylated PrP^{Sc} forms, reveals that only sample RV21 shows a ratio between di- and monoglycosylated PrP^{Sc} significantly higher than 1. Therefore, samples RV12, RV13, RV16 and RV20 are classified as type 2A, while sample RV21 is classified as type 2B. A ratio between di- and monoglycosylated PrP^{Sc} significantly lower than 1 (pattern A) also characterizes the PrP^{Sc} type 1 samples from sporadic TSEs subtypes (Figure 1B).

Figure 2 shows several type 1 (Figure 2A) and type 2 (Figure 2B) samples run side by side in a gel electrophoresis

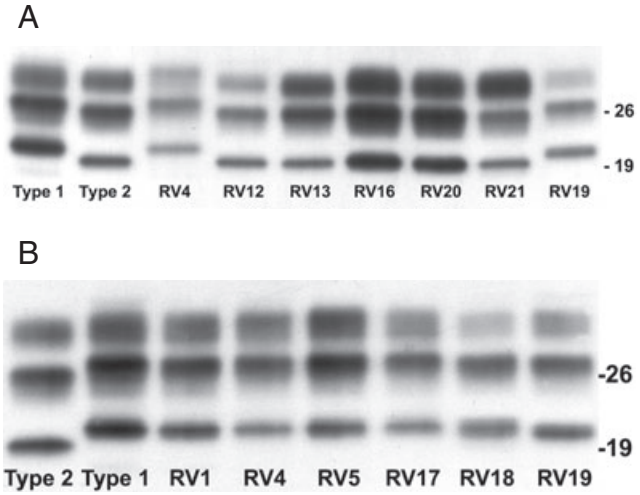

Figure 1. Immunoblot analysis of PrP^{Sc} extracted from frontal cortex homogenates of blindly coded human TSE brain samples. All samples were treated according to given instructions (see Materials and Methods) and were separated in a conventional gel electrophoresis system [12% Tris–glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 6.5-cm separation distance]. Panel **A** shows an example of the first analyses, while panel **B** illustrate an example of the second analyses, during which all cases identified as having the same PrP^{Sc} type were run together.

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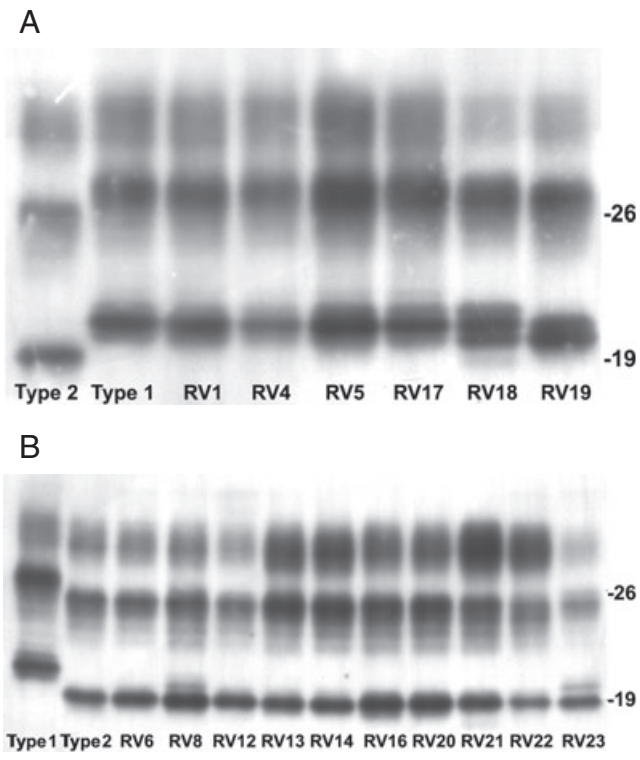

Figure 2. Immunoblot analysis of PrP^{Sc} extracted from frontal cortex homogenates of blindly coded human TSE brain samples. All samples were treated according to given instructions (see Materials and Methods) and were separated in a high-resolution gel electrophoresis system [15% Tris–gylcine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15-cm separation distance]. The samples, previously analyzed in a conventional gel electrophoresis system (see Figure 1), were diluted or concentrated to even the signal intensities of the samples and were loaded either onto gel 1 (**A**) or gel 2 (**B**) according to their migration pattern (ie, type 1 or type 2) determined in the conventional gel electrophoresis system.

system allowing a greater band separation (ie, separating gel of 15 cm). There are visible but subtle differences in the migration rates of the unglycosylated band, which, in sample RV18 and even more in sample RV19, migrated slightly faster than in the other RV samples classified as type 1 (Figure 2A). The subtle faster migration of sample RV19 is already seen with the conventional gel electrophoretic system (Figure 1B). Furthermore, samples RV8 and RV23 include an additional band migrating slightly more slowly that is not seen in the other RV type 2 samples (Figure 2B). Thus, according to Notari *et al* (19), samples RV18 and RV19 are classified as type 1 of the VV1 subtype, and samples RV8 and RV23 as type 2A of the MV2 subtype with kuru plaques.

Classification by participant laboratories

Participants were asked to classify their samples according to the type1/type 2A/2B scheme by Parchi *et al* (22, 23) (Figure 1) and subsequently to try to identify subtypes within the two sample groups according to Notari *et al* (19) (Figure 2). All gels were also evaluated independently in laboratories 1 and 2 by two additional reviewers in each center. The technical quality of the gels submitted was variable, ranging from acceptable to excellent. Classification by the participants according to the type 1/type 2A/2B scheme is shown in Table 1. Despite the variable quality of the gels, the agreement was excellent. Indeed, in 14 out of 16 samples, the same classification was given by all participants. The only exceptions to the full agreement concerned samples RV18 and RV23. Sample RV18 was given a type 1 pattern by five participants, while two found a mixture of types 1 and 2A in it, with type 1 giving the predominant signal in both instances. More precisely, one laboratory obtained an unquestionable PrP^{Sc} types 1 and 2 co-occurrence (Figure 3), whereas another one found a minor type 2 component also, but only after running the sample in the electrophoretic system with increased separation (Fig. 2A).

Regarding sample RV23, six laboratories typed it as 2A, whereas the remaining one classified the sample as type 1. Given that the latter was the only definite discrepancy obtained, the possibility of

Table 1. Classification of samples by participant laboratories according to the type1/type 2A/2B scheme. Abbreviations: C = classification by supplying laboratories; vCJD = variant CJD.

Lab	RV1	RV4	RV5	RV6	R8	RV12	RV13	RV14	RV16	RV17	RV18	RV19	RV20	RV21	RV22	RV23
1	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	2A
2	1	1	1	2A	2A	2A	2A	2A	2A	1	**1 + 2**	1	2A	2B	2B	2A
3	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	1*
4	1	1	1	2A	2A	2A	2A	2A	2A	1	**1 + 2**	1	2A	2B	2B	2A
5	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	2A
6	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	2A
7	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	2A
C	1	1	1	2A	2A	2A	2A	2A	2A	1	1	1	2A	2B	2B	2A
	MV	MV	MM	MMT	MV	MMT	VV	VV	MMC	MM	VV	VV	MMC	vCJD	vCJD	MV

*The sample was typed 2A on a second delivered piece of tissue from the same case (cf. text) suggesting mislabeling, transcription errors, sample mix-up or similar events.

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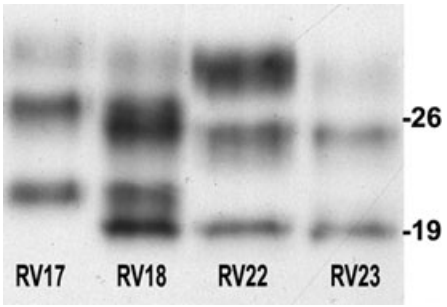

Figure 3. Western blot analyses (laboratory 4) on case RV18 showing a co-occurrence of PrP^{Sc} types 1 and 2. One PrP^{Sc} type 1 (RV17) and two PrP^{Sc} type 2 samples (RV22 and RV23) are included for comparison.

a sample mislabeling or similar mistake during shipment was taken into consideration. Thus, a second sample 23 was sent blindly, together with two other samples, to the laboratory. This second time, the sample was typed consistently with all other laboratories (ie, type 2A).

A full consensus between laboratories was seen with respect to the visible ratio of the amount of the mono- and diglycosylated bands. Despite the fact that no specific instructions were given for the densitometric analyses of bands and that each laboratory used its own method and equipment for the densitometric analyses (Table 2), all laboratories correctly classified samples RV21 and RV22 as a type 2B. Noteworthy, however, occasionally (in 5 out of 98 analyses), samples from sCJD cases also showed a higher intensity of the diglycosylated PrP^{Sc} band compared with the monoglycosylated one, although this was quantitatively less significant than in vCJD cases except for one MM2 case (RV12, laboratory 5). However, none of the sporadic TSE samples had a significantly higher amount of the diglycosylated band with respect to both mono- and unglycosylated bands, which instead was a constant feature of the vCJD samples in all analyses. In particular, the calculated ratio between di- and monogylcosylated as well as di- and unglycosylated bands in vCJD samples was always higher than 1.3 and 1.9, respectively.

Table 3. Subclassification of type 1 and type 2 samples.

Laboratory	RV8	RV17	RV18	RV23
1	–	–	–	MV2
2	MV2	VV1	VV1 + 2	MV2
3	MV2	–	–	–
5	–	–	VV1	–

At variance with the type 1/type 2A/2B classification, there was less agreement when the classification in subtypes was attempted, as shown in Table 3. First, most laboratories made no attempt to classify the samples by this approach, probably because of failure to detect the subtle differences on which it is based. The variable quality of the gels and the fact that only one laboratory reanalyzed the samples with an electrophoretic system of higher resolution may also have contributed. Three laboratories made the attempt to recognize the VV1 and MV2 cases with kuru plaques that, according to Notari *et al* (19) show a slightly different migration pattern compared to the other type 1 and type 2 cases, respectively. In particular, the doublet which often characterize the MV2 cases with kuru plaques should also be distinguished by a PrP^{Sc} types 1

Table 2. PrP^{Sc} glycotype analyses (in %) by participating laboratories. Values refer to the relative % of unglycosylated (upper), monoglycosylated (middle), and unglycosylated (lower) PrP^{Sc} isoforms based on densitometric analyses.

Lab	RV1	RV4	RV5	RV6	RV8	RV12	RV13	RV14	RV16	RV17	RV18	RV19	RV20	RV21	RV22	RV23
1	17.5	26.5	25.4	14.7	23.2	13.2	14.4	29.7	20.8	20.2	10.2	14.0	15.6	**59.6**	**60.9**	16.2
	47.1	47.6	43.8	37.0	42.1	45.2	44.2	37.8	42.3	35.2	45.4	44.3	41.1	**23.7**	**24.9**	33.3
	35.4	25.9	30.8	26.3	34.7	41.6	41.4	32.5	36.9	44.6	44.4	41.7	43.3	**16.7**	**14.2**	50.5
2	33.4	37.6	36.8	29.0	25.9	29.6	38.6	39.8	35.5	33.4	19.8	27.2	33.7	**57.5**	**59.4**	33.3
	43.1	44.0	43.3	45.2	42.7	44.4	43.5	42.8	40.6	46.2	49.5	44.9	42.4	**30.3**	**31.3**	37.9
	23.5	18.4	19.9	25.8	31.4	26.0	17.9	17.4	23.9	20.4	30.7	27.9	23.9	**12.2**	**9.3**	28.8
3	15.3	30.9	25.9	17.6	21.3	20.1	35.0	27.4	27.7	19.0	16.9	25.1	17.7	**52.5**	**51.8**	24.3
	46.5	40.1	33.0	47.3	40.7	38.5	39.5	36.6	40.8	41.0	45.5	36.7	34.5	**24.1**	**26.9**	33.6
	38.2	29.0	41.1	35.1	38.0	41.4	25.5	36.0	31.5	40.0	37.6	38.2	47.8	**23.4**	**12.3**	42.1
4	20.4	25.6	26.0	25.3	21.1	24.8	30.9	29.3	33.9	18.5	10.0	23.7	32.5	**48.8**	**47.2**	19.9
	44.0	48.5	43.9	37.8	44.6	43.4	40.8	38.2	43.4	48.6	47.1	46.8	41.8	**32.9**	**34.2**	43.4
	35.6	25.9	30.1	36.9	34.3	31.8	28.3	32.5	22.7	32.9	42.9	29.5	25.7	**18.3**	**18.6**	36.7
5	29.5	31.1	30.5	33.1	32.1	45.9	39.5	36.9	35.6	17.1	24.3	36.5	34.6	**52.4**	**46.0**	27.1
	39.2	42.9	38.3	40.5	33.7	38.3	40.9	39.7	42.4	66.5	44.6	39.3	45.8	**30.2**	**30.6**	37.4
	31.3	26.0	31.1	26.3	34.2	15.7	19.5	23.4	22.1	16.3	31.1	24.2	19.6	**17.3**	**23.4**	35.5
6	21.5	22.5	29.7	32.3	35.2	34.1	31.9	33.8	29.4	27.6	17.7	18.4	33.7	**52.9**	**51.6**	27.7
	45.3	45.2	39.4	33.4	38.5	31.3	28.0	27.7	29.8	43.8	43.0	44.5	26.0	**24.5**	**27.4**	30.0
	33.2	32.3	30.9	34.3	26.3	34.6	40.1	38.5	40.8	28.6	39.3	37.1	40.3	**22.6**	**21.0**	42.3
7	27.7	29.3	27.0	17.2	29.4	14.3	33.9	33.2	20.6	25.1	13.3	16,6	18.9	**46.7**	**50.9**	22.2
	53.8	44.7	45.6	46.7	46.3	44.1	43.8	46.3	42.4	45.1	40.3	44.2	46.9	**34.2**	**35.5**	43.7
	18.5	27.0	27.4	36.1	24,3	41.6	22.3	20.5	37.0	29.8	46.4	39.2	34.2	**19.1**	**13.6**	35.1

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and 2 co-occurrence, and this is based on a faster migration of the upper band of the doublet (~20 kDa) compared with type 1 (~21 kDa).

Full recognition of the three cases included in the series was achieved by one laboratory, whereas one out of two MV2 cases or one VV1 case was recognized by each of the other three. Only the laboratory which identified the three cases correctly reanalyzed the samples with an electrophoretic system of higher resolution. Finally, none of the laboratories interpreted the recognized doublet as a PrP^{Sc} types 1 and 2 co-occurrence.

DISCUSSION

In the apparent absence of a disease-specific nucleic acid, the molecular diagnosis of prion diseases must rely on protein studies. Besides PrP^{Sc} detection, which is required for a definite diagnosis of prion disease, PrP^{Sc} isotype analysis has proven to be extremely useful in the molecular classification and differential diagnosis of CJD and is likely to continue to play a major role in the investigation of human prion diseases. Differences in PrP^{Sc} cleavage, possibly reflecting differences in protein conformation and glycosylation, have been correlated with disease phenotype and, together with *PRNP* genotype, comprise the basis for current molecular classification of human TSEs (19, 25).

Despite its importance and the widespread use among laboratories involved in TSE surveillance worldwide, and the existence of several research studies where PrP^{Sc} typing results have been generated in different laboratories, there are no published results on the issue of inter-laboratory reproducibility of molecular TSE typing.

The data reported here concern a collaborative study involving all the major groups involved in human TSE diagnosis in Europe, to assess the robustness and transferability of methods for the classification of samples from TSE brains based on the analyses of physicochemical properties of PrP^{Sc} as proposed by Parchi *et al* (22, 23) and Notari *et al* (19). More specifically, the aims of the study included the analysis of inter-laboratory reproducibility of assessment of types 1, 2A and 2B PrP^{Sc}, and of VV1 and MV2 sCJD subtypes recognition.

Concerning the first objective, the assessment of PrP^{Sc} types 1, 2A and 2B, the results obtained were in agreement for all laboratories with two exceptions. First, two laboratories found the co-occurrence of both PrP^{Sc} types 1 and 2 in one sample, and second, one laboratory classified as type 1 one sample which was classified as type 2A by all the other laboratories. We attributed the first discrepancy to a mishap in case selection, as one of the selection criteria of this study was to exclude such mixed type 1 and type 2A cases. The fact that only a few laboratories detected the minor type 2 component in the sample is well explained by the well-known variability in distribution of these two PrP^{Sc} subtypes in brains where they co-occur.

As far as the second discrepancy is concerned, we believe that it can be attributed to a mislabeling or an involuntary exchange of samples as the case was typed correctly when it was examined blindly the second time using a newly dispatched sample. The present results demonstrate that the inter-laboratory reproducibility of human PrP^{Sc} typing based on the type 1, type 2 scheme is high and close to full agreement. The same conclusion can be drawn for the type 2A/2B distinction, although our results suggest a better operational definition for pattern 2B. Indeed, our results indicate that, when dealing with samples from the cerebral cortex, the glycopattern 2B should be given only to those samples in which the diglycosylated band is the most abundant and the ratios between the di- and the monoglycosylated and the di- and the unglycosylated PrP^{Sc} bands are higher than 1.3 and 1.9, respectively.

Concerning the second objective, the recognition of CJD subtypes such as VV1 and MV2 sCJD based on PrP^{Sc} typing alone, it appears much less satisfactory. However, some methodological consideration needs to be addressed in this respect. First of all, as described by Notari *et al* (19), the identification of such cases, which is based on subtle differences in electrophoretic mobility, is best accomplished by using a gel electrophoresis system with high resolution. Given that such system is laborious and not routinely used in all laboratories, in the present study, it was our aim to verify whether such more subtle differences in migration could be reproduced among laboratories using standard conditions. To this aim, we asked to identify all differences in gel migration using a standard apparatus, leaving the possibility to run the longer gels with higher resolution as a further option. The results obtained indicate that the distinction of further subtypes within the type 1 and type 2A samples, based on the pattern of electrophoretic mobility of PrP^{Sc} alone, is not reliable, at present, when comparing data from different laboratories using only short standard gels. Nevertheless, it is noteworthy that the laboratory in which such distinctive patterns were originally described blindly typed all samples correctly. The distinction appears therefore reproducible and real, but, at present, is less transferable in the inter-laboratory setting than the type 1, 2A and 2B classification scheme.

Current difficulties in CJD post-mortem molecular diagnostics relate to the finding of cases with PrP^{Sc} type co-occurrence in the same brain (25, 31, 11). Thus, the reason why we deliberately choose to exclude such cases might represent a significant drawback of the present study and, therefore, requires an explanation. At least two significant reasons led us to make such decision. First of all, as also demonstrated by case RV18 in the present study, the distribution of each protein type in brains showing the co-occurrence of PrP^{Sc} types is highly variable and still largely undetermined. As a consequence, an inter-laboratory study to assess reproducibility may require the analysis of brain homogenates rather than the intact brain tissue. Even more importantly, there are significant controversial issues in the current literature to be solved concerning the definition and correct interpretation of biologically relevant co-occurrences of PrP^{Sc} fragments, before such topic should be addressed in the inter-laboratory setting (20, 29, 37).

A number of conclusions can be drawn from the present study. First and foremost, all seven laboratories involved correctly identified the two type 2B samples from vCJD cases. In practical terms, for human CJD surveillance in Europe, this is the most important result. Furthermore, there were no differences in the distinction of type 1 and type 2 samples with one exception, which eventually was identified as a case with type 1 and type 2 coexistence. This shows that the techniques used in this study are very robust and therefore can be considered as a reliable basis for diagnostic and epidemiologic purposes. The subtle further distinction of subtypes among type 1 and type 2 groups requires high-sensitivity gel electrophoresis protocols that are unsuitable

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for routine diagnostic needs and must be reserved for research investigations. Further research is also necessary to explore the significance of co-occurrence of type 1 and type 2 PrP^{Sc} within one brain.

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