Factors intrinsic and extrinsic to blood hamper the development of a routine blood test for human prion diseases

Hanin Abdel-Haq

Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena, 293, 00161–Rome, Italy

Development of numerous advanced techniques in recent years have allowed detection of the pathological prion protein (PrP\text{\tiny TSE}), the unique marker of transmissible spongiform encephalopathies (TSEs, or prion diseases), in the blood of animals and humans; however, an ante mortem screening test that can be used for the routine diagnosis of human prion diseases remains unavailable. A critical, analytical review of all the diagnostic assays developed to date will allow an evaluation of progress in this field and may facilitate the identification of the possible reasons for this delay. Thus, in this review, I provide a detailed overview of the techniques currently available for detecting PrP\text{\tiny TSE} and other markers of the disease in blood, as well as an analysis of the significance, feasibility, reliability and application spectrum for these methods. I highlight that factors intrinsic and extrinsic to blood may interfere with the detection of PrP\text{\tiny TSE}/prions, and that this is not yet taken into account in current tests. This may inspire researchers in this field to not only aspire to increase test sensitivity, but also to adopt other strategies in order to identify and overcome the limitations that hamper the development of a successful routine blood test for prion diseases.

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative diseases that are transmissible from one individual or species to another by different infection routes (Prusiner, 1998).

The infectious agent of TSEs remains undefined; however, a key event in prion disease pathogenesis is thought to be the conversion of the cellular prion protein (PrPC) to the partially protease-resistant form (PrP\text{\tiny Sc}) and its deposition in the central nervous system (Bolton et al., 1982; Kocisko et al., 1996). PrP\text{\tiny C} is an ubiquitous glycoprotein that, when converted to PrP\text{\tiny Sc}, acquires detergent insolubility (Gabizon et al., 1987), protease resistance (Bolton et al., 1982; Oesch et al., 1985), higher beta-sheet content (43 % vs 3 %) (Caughey et al., 1991; Gasset et al., 1993; Pan et al., 1993), a tendency to form amyloid fibril deposits in the brain (Masters & Richardson, 1978) and an altered glycosylation pattern (Rudd et al., 1999; Pan et al., 2005a; Triantaphyllidou et al., 2006).

Although the natural route of TSE transmission has not yet been determined, the oral route, which is the basis for the transmission of Kuru (Collinge et al., 2006), transmissible mink encephalopathy (TME) (Bessen & Marsh, 1994), bovine spongiform encephalopathy (BSE) and variant Creutzfeldt–Jakob disease (vCJD), is the route of infection that was ascertained (Thackray et al., 2003). However, because transmission by blood has been demonstrated experimentally from animal to animal for some TSEs (Brown et al., 1998; Houston et al., 2000; Holada et al., 2002; Hunter et al., 2002; Cervenakova et al., 2003), and is probable naturally from human to human for vCJD (reviewed by Hewitt et al., 2006; UK Health Protection Agency, 2007; Chohan et al., 2010), blood can also be considered an efficient route of infection.

Since no cure is available, and as blood is essential for transfusions and is the starting material for various life-saving, plasma-derived products, the safety of public health is threatened by blood transmission of TSE. Therefore, effective measures to protect against its transmission are greatly needed. Early diagnosis of prion diseases with a reliable blood screening test would offer a primary precautionary measure.

In addition to enabling the exclusion of infected blood from transfusions and plasma manufacturing, thereby preventing further transmission, an effective blood test would also allow the identification of infected but asymptomatic individuals. Presymptomatic diagnosis is extremely important for effective treatment, as it would enable therapeutic intervention at an early stage of the disease, before the appearance of definitive clinical signs and permanent brain damage. Such treatment may be possible because numerous anti-prion compounds that have been tested in animal models showed efficacy when they were administered before or upon infection, rather than when they were administered...

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after the appearance of clinical symptoms, regardless of the
route of infection (Forloni et al., 2002; Doh-Ura et al., 2004;
Kocisko et al., 2006a, b; Abdel-Haq et al., 2009; Chung et al.,
2011; Relaño-Ginés et al., 2011; Ryoo et al., 2011; Sim,
2012). In addition, early diagnosis may aid in the develop-
ment of specific treatments based on the relationship
between disease symptoms and a potential diagnostic marker
profile. In the light of these possibilities, therapy for indi-
viduals with early (ascertained) diagnosis might be achieved
with some drugs, either alone or in combination, that have
been shown to have a high anti-prion activity against human
strains. This may include drugs such as pentosan polysulfate,
which was recently shown to exert some effect on the
accumulation of both protease-resistant prion protein
(PrPres) and oligomeric PrP, even in the brains of some
CJD patients at the clinical stage of the disease (Honda et al.,
2012). Alternatively, therapy may be achieved by utilizing
drugs that are currently used for other diseases that cause
symptoms similar to those in the early stage of prion diseases,
that may show similar efficacy against prions, or by
developing new targeted drugs. Such early intervention
may limit brain damage, hence delaying the appearance of
clinical symptoms, and perhaps also reducing their severity,
thus improving the patient’s quality of life and resulting in
cost savings (Murman, 2012). This could be possible because
the early symptoms of such diseases are usually very mild and
common to other diseases because they are likely due to
reversible functional disorders, which if not treated promptly,
would become definitive due to brain impairment and pro-
gressively worsen until dementia (Sheinerman & Umansky,
2013). However, although numerous advanced methods that
have recently been developed are able to detect PrPTSE (the
unique marker of prion diseases) in the blood of animals
and humans, no routine blood screening test is available yet.

Here, I provide a detailed overview of the current methods
for detecting PrPTSE and other possible markers of the
disease in blood, coupled with an analysis of the signifi-
cance, feasibility, reliability and application spectrum of
these methods, with a view to identifying the reasons for
the delay in the development of a routine blood test.

Tests based on the detection of PrPTSE

Because PrPS is the pathognomonic marker of TSEs, almost
all the blood tests developed to date are based on the
detection of this protein. All of the blood tests described here
(Table 1) have succeeded in detecting exogenous and
endogenous PrPTSE in blood components obtained from
different species (humans and animals) during the early or
late stages of the disease.

It has been reasoned that in relation to the infectivity titre
in blood (approximately 10–100 IU ml⁻¹ at the terminal
stage), the concentration of PrPTSE in blood would be
extremely low (<1 fg ml⁻¹) (reviewed by Brown et al.,
2001a; Soto, 2004). Therefore, additional enrichment steps
would be required to allow detection by conventional
immunoassays. Based on this rationale, several authors
have exploited the nucleation principle (Prusiner, 1982,
1998) in the development of their blood screening tests,
the prototype of which is the protein misfolding cyclic
amplification (PMCA) method that was first developed by
Saborio et al. (2001).

Amplification-based approaches

PMCA

PMCA exploits the fact that when PrPC is incubated with a
PrPTSE template, it is converted to PrPTSE. This allows
amplification of minute amounts of PrPTSE in the presence
of an excess of PrPC, which form new aggregates that are
then dissociated by sonication into smaller units, which, in
turn, act as seeds for further replication.

Due to this amplification property, PMCA was used by
Castilla et al. (2005) to detect PrPTSE in the blood (buffy
coyt) of terminally ill hamsters and mice, with 89% and
80% sensitivity, respectively. This experiment showed that
1 ml of scrapie-infected hamster blood contains roughly
0.1–1 pg of PrPTSE. This discovery prompted Saa et al.
(2006) to attempt the detection of PrPTSE in the buffy coat
of hamsters infected intraperitoneally with the 263K prion
strain of scrapie, after fractionation at different time points
during the incubation period of the disease. As expected,
PMCA allowed detection of PrPTSE during the clinical and
preclinical stages of the disease with 80% and 60% sensitiv-
ity, respectively, and 100% specificity (Saa et al., 2006).

This technique was also shown to be highly efficient for the
detection of the PrPTSE of other scrapie strains and in other
blood components. It allowed detection of PrPTSE in the
plasma and buffy coat of hamsters infected via intracerebral
or oral inoculation with the Sc237 prion strain of scrapie
during the terminal (100% sensitivity) and asymptomatic
(<100% sensitivity) stages of the disease (Murayama et al.,
2007) and in white blood cells obtained from sheep
naturally infected with scrapie (Thorne & Terry, 2008; Lacroix
et al., 2012). It also allowed detection of PrPTSE in
whole blood collected from CD1 mice that were experi-
mentally infected with the Rocky Mountain Laboratory
(RML) prion strain of scrapie, and in whole blood from
uninfected CD1 mice that was exogenously spiked with RML-
infected brain homogenate (Tattum et al., 2010a). Further-
more, very recently, PMCA was also used to detect PrPTSE
in the white blood cells and buffy coat from BSE-infected sheep
and vCJD-infected primates (cynomolgus monkey) during
the preclinical and clinical stages of the disease, and from
vCJD-affected patients (Lacroix et al., 2014).

PMCA was able to amplify PrPTSE in whole blood, buffy
coyt, plasma and white blood cells from hamsters, sheep,
monkeys, mice and humans infected with 263K, Sc237,
PG127, BSE, RML or vCJD. The sensitivity of tests em-
ploying PMCA for PrPTSE detection is between 60% and
100%. The detection sensitivity in plasma is lower than that
in buffy coat, and it is 50% lower in whole blood, due to its
inhibitory nature. PMCA is time-consuming process and has a marked disadvantage: a high risk of the spontaneous generation of protease-resistant PrP. It is also limited in its adaptation for use with different species, because of its dependence on the choice of the substrate for amplification, although this now seems to have been overcome (Thorne & Terry, 2008; Tattum et al., 2010a).

**Plasminogen-based assay**

To overcome the inhibitory effect of various factors present in blood, which significantly reduce the amplification efficiency of PMCA (Tattum et al., 2010a), a PrP<sub>TSE</sub>-capturing step was introduced before the PMCA (Segarra et al., 2013). Since plasminogen is a well-known prion protein ligand, it was used to capture PrP<sub>TSE</sub> in blood samples. This approach reached almost 100% efficiency, without any false positives, and allowed detection of PrP<sub>TSE</sub> in plasma obtained from one sheep at the preclinical phase of scrapie (127S strain, a mouse-adapted PG127 isolate) after only two PMCA rounds and in Buffy coat after only one round. Moreover, this assay showed 100% specificity and sensitivity when it was applied to white blood cells from naturally scrapie-infected sheep.

However, because the capturing step is not selective for PrP<sub>TSE</sub>, plasminogen-coated nanobeads may also capture PrP<sub>C</sub>. However, since PrP<sub>C</sub> is the essential substrate for PrP<sub>TSE</sub> amplification and must be present in excess, this is not a limitation for PMCA. Eventual competition between PrP<sub>C</sub> and PrP<sub>TSE</sub> for capture on the coated beads may lead to PrP<sub>TSE</sub> loss, which would affect the PMCA amplification ratio. Yet, this assay was able to detect PrP<sub>TSE</sub> in as little as 25 μl of Buffy coat and 500 μl of plasma collected during the preclinical stage of the disease. It also achieved almost 100% capture efficiency in serial dilutions (up to 10<sup>-8</sup>) of human vCJD brain homogenate spiked into normal human plasma, which corresponds to the sensitivity required for the detection of PrP<sub>TSE</sub> in asymptomatic subjects. Therefore, it has been proposed that this assay be used as a confirmatory test for the presence of PrP<sub>TSE</sub> in blood samples judged positive in large-scale screening tests (Segarra et al., 2013).

This method is based on amplification of PrP<sub>TSE</sub> and detection by Western blotting after capture on plasminogen-coated nanobeads. It was able to capture PrP<sub>TSE</sub> from Buffy coat, plasma and white blood cells obtained from sheep infected with 127S, and from human plasma spiked with vCJD. It required low volumes and showed 100% sensitivity, specificity and capture efficiency. However, it is a multistep, time-consuming process. Its main disadvantage is that the capturing step is not selective for PrP<sub>TSE</sub>; therefore, competition between PrP<sub>C</sub> and PrP<sub>TSE</sub> for capture on the beads may affect the amplification ratio due to loss of PrP<sub>TSE</sub>.

**Surrounding optical fibre immunoassay (SOFIA)**

Attempts have been made to improve both the sensitivity and specificity of the PMCA assay, while reducing the possibility of PrP<sub>C</sub> self-aggregation. An interesting approach that has been recently described involves a combination of PMCA and a SOFIA (Rubenstein et al., 2010). Using this approach, the number of cycles necessary for the amplification of PrP<sub>TSE</sub> could be limited without obtaining PMCA-related false-positives after a particular number of amplification cycles; in these cases, PrP<sub>TSE</sub> also spontaneously formed in the control samples. This assay detected PrP<sub>TSE</sub> in the plasma of scrapie-infected sheep and chronic wasting disease (CWD)-infected white-tailed deer during the clinical and preclinical stages of the disease, without requiring proteinase-K (PK) digestion. SOFIA is a sandwich ELISA in which Rhodamine Red X coupled to streptavidin is used instead of an enzyme conjugate, which considerably increases the assay sensitivity. However, this reduces its widespread use.

SOFIA is based on the immunoprecipitation (IP) of PrP<sub>TSE</sub> and its detection in a sandwich ELISA after amplification. This method allowed amplification of PrP<sub>TSE</sub> in plasma from sheep and white-tailed deer infected with scrapie and CWD, respectively. The detection sensitivity of SOFIA was increased by using Rhodamine instead of an enzyme conjugate, although this change limited its widespread use. It also has a multistep, time-consuming method.

**Am-A-FACCT assay**

To overcome some of the risks associated with PMCA while taking advantage of its underlying principles, aggregation-specific ELISA (AS-ELISA), which was initially developed to detect brain PrP<sub>TSE</sub> aggregates (Pan et al., 2005b), was combined with an in vitro PrP<sub>TSE</sub>-amplification step, similar to that used in PMCA, catalysed by T7 RNA polymerase (FACTT) (Chang et al., 2007). This assay was called Am-A-FACCT and was able to detect aggregates of PrP<sub>TSE</sub> during the terminal and asymptomatic phases of the disease in the plasma of CD1 mice infected intraperitoneally with the ME7 prion strain of scrapie, and of mule deer orally infected with CWD. This assay showed 100% detection sensitivity and specificity at a late, but still asymptomatic, stage of the disease, and 50% sensitivity and 100% specificity at a very early stage of the disease, when assaying plasma from infected mice. In this assay, plasma is mixed with healthy brain homogenate and then subjected to amplification. Then, newly formed PrP<sub>TSE</sub> aggregates are captured by an antibody specific for aggregates. These are finally detected using a biotin-conjugated DNA template. Transcription of the DNA template into RNA is followed by the addition of an RNA-intercalating dye, and then the intensity of the emitted light is measured.

This technique is based on PrP<sub>TSE</sub> amplification and capture of the newly formed PrP<sub>TSE</sub> aggregates by a specific antibody. Using this method, it was possible to amplify PrP<sub>TSE</sub> in plasma from mice and mule deer infected with ME7 and CWD, respectively. The detection sensitivity was 100% at the preclinical stage of the disease and 50% at an even earlier stage, and the specificity was 100%. Given that
Table 1. Summary of all the techniques currently available for detecting PrP\textsuperscript{TSE} and other markers of the disease in blood

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<th>TSE strain</th>
<th>Disease phase</th>
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BC, Buffy coat; WB, whole blood; WBC, white blood cells.

*Spiked material (exogenous).
†Either endogenous or exogenous.
‡Failure in the detection of PrP<sup>TSE</sup>.
§Either detection or failure.
this is a multistep test based on PrPTSE amplification and PrPTSE aggregate capture, the possibility of errors that generate false positives is markedly increased.

**Real-time quaking-induced conversion (RT-QuIC)**

Other researchers have attempted a modified PMCA approach, known as enhanced quaking-induced conversion (e-QuIC) (Orru et al., 2011). This approach combines QuIC, which can be as sensitive as an in vivo bioassay and in which prions induce the polymerization of recombinant PrPC (rPrPC) into amyloid fibrils by applying cycles of shaking and rest (Atarashi et al., 2008; Orru et al., 2009), with specific IP of PrPSc using the 15B3 antibody (Korth et al., 1997; Biasini et al., 2008, 2009). At first, this approach employed the IP-standard (S)-QuIC, which was able to detect PrPTSE in either plasma from scrapie-infected hamsters at the clinical stage of the disease or human plasma spiked with dilutions of hamster scrapie or human vCJD brain homogenate in which approximately 1–10 fg of PrPTSE could be detected. Subsequently, a more sensitive format, RT-QuIC was used, with substrate replacement. In addition to the IP step, this approach uses multiwell plates and thioflavin T-based fluorescence detection of the prion-seeded amyloid. This IP-RT-QuIC approach, termed the enhanced RT-QuIC (e-QuIC) assay, is several orders of magnitude more sensitive than the other tests. It allowed detection of PrPTSE in the plasma of scrapie-infected hamsters at the preclinical stage of the disease, and PrPTSE in human plasma spiked with high dilutions (up to $10^{-14}$ or approximately 2 ag ml$^{-1}$ PrPTSE) of human vCJD brain homogenate. Moreover, this approach showed $>80\%$ sensitivity and 100% specificity in discriminating between sporadic CJD (sCJD) and non-CJD patients, by analysing their cerebrospinal fluid (CSF) (Atarashi et al., 2011).

The developers of this approach suggested that this promising blood test could be used for the diagnosis of TSEs in humans and animals. The RT-QuIC assay was shown to be applicable for the detection of PrPTSE in the whole blood of CWD-infected cervids and TME-infected hamsters, during both the preclinical and clinical phases of the disease, with approximately 94% sensitivity and 100% specificity (Elder et al., 2013). Moreover, it has been shown that limited cycles of freeze–thawing of the blood samples enhances the sensitivity of blood-borne prion detection by RT-QuIC during the preclinical phase of the disease (Elder et al., 2013). This increased sensitivity may be due to the damaging effect of the thermal shock on the membranes of infected blood cells, which releases the intracellular components, including prions, thus increasing the in vitro nucleation and consequently the detection of PrPC-converting activity (Elder et al., 2013).

However, the Rt-QuIC assay has an important limitation, namely, that the best rPrPC substrate for amplification is not always the same as the prion/PrPres being assayed. It is thought that this may be due to condition-dependent factors, such as differences in the propensity to form an amyloidogenic precursor, or an intermediate that may be totally different from the native rPrPC that interacts more favourably and efficiently with prion seeds. Alternatively, it may be due to the occurrence of off-pathway changes that may promote spontaneous, prion-independent fibril formation (false positives) or remove rPrPC from the assembly component pool (Orru et al., 2011).

RT-QuIC is based on amplification of PrPTSE after IP with a specific antibody. It has been used to detect PrPTSE in whole blood, plasma and serum from hamsters infected with 263K, and cervids infected with CWD or TME, as well as in human plasma spiked with vCJD. It displayed $>94\%$ sensitivity and 100% specificity. It detected exogenous human PrPvCJD at a $10^{-14}$ dilution in human plasma. However, it is time consuming, and its main disadvantage is choosing the rPrPC substrate, which has to be compatible with the assayed PrPTSE. It also shows a high risk for false positives.

**Capture-based approaches**

**ELISA**

Because the PMCA and RT-QuIC amplification ratios are both influenced by the concentration of the substrate (PrPC), some researchers have adopted other approaches that specifically capture and concentrate the disease-associated PrPTSE from blood fractions before employing detection by standard methods. IP with anti-PrP antibodies is one of the most commonly used approaches, which is described here for the assay developed by Tatum et al. (2010b). This assay allowed the detection of $2 \times 10^6$ LD$_{50}$ units ml$^{-1}$, equivalent to detection at a 25 000-fold dilution of human vCJD brain homogenate spiked into healthy whole human blood, and 750 LD$_{50}$ units ml$^{-1}$, equivalent to detection at a 2.5 million-fold dilution of 8 ml of whole blood spiked with human vCJD brain homogenate (Tatum et al., 2010b). In this test, PK was replaced with thermolysin, a metalloprotease enzyme that digests PrP$_C$ but leaves PrPTSE intact.

This assay is based on immunodetection of PrPTSE in a standard ELISA after IP with a specific antibody. The assay detected PrPvCJD in whole human blood spiked with human vCJD-infected brain homogenate with high sensitivity and specificity; however, it has not yet been applied to endogenously infected samples.

**Solid-state binding matrix**

In another assay, instead of concentrating PrPTSE by IP with anti-PrP antibodies, PrPTSE was concentrated from human whole blood by capturing it on stainless steel beads before immunodetection using a solid-state capture matrix-ELISA (Edgeworth et al., 2011). This assay was shown to be more efficient than IP with antibodies, and allowed the detection of PrPTSE in human whole blood spiked with high dilutions (up to $10^{-10}$) of vCJD-infected brain homogenate, and in the whole blood of 15 of 21 symptomatic patients with vCJD.
with 71.4 % sensitivity and 100 % specificity. This assay was recently validated by the same research group, using a larger number of samples, including healthy and potentially cross-reactive patient populations (Jackson et al., 2014). This validation study not only confirmed the previously determined specificity and sensitivity of the assay for vCJD infection in blood, but also identified 2 of 105 patients who were positive for sCJD, indicating that this assay was also sensitive for significantly lower levels of prion infectivity, such as those associated with sCJD (Jackson et al., 2014). However, this assay is still limited by its insufficient sensitivity for the detection of asymptomatic individuals with vCJD who are subclinical carriers, or at the preclinical stage of the disease.

This method is based on the capture of PrP TSE on stainless steel beads before immunodetection in a special ELISA. It detected PrP TSE in human whole blood spiked with up to a 10^{-10} dilution of human vCJD-infected brain homogenate, and in the whole blood of two patients with sCJD and 15 symptomatic patients with vCJD, with 71.4 % sensitivity and 100 % specificity. The main disadvantage of this assay is its insufficient sensitivity for the detection of asymptomatic, TSE-infected individuals.

**PrP-aggregate detection-based approaches**

**Ligand-based immunoassay and surface fluorescence intensity distribution analysis (sFIDA)**

In another approach based on a rapid, ligand-based immunoassay, PrP TSE was captured by a polyanionic ligand before its detection in PBMCs prepared fromuffy coat obtained from clinical and asymptomatic sheep that were naturally or experimentally infected with prions (Terry et al., 2009; Edwards et al., 2010). In this assay, PrP TSE was detected in the PBMCs of 55 % and 71 % of scrapie- and BSE-infected animals, respectively, but was not detected in the plasma (Terry et al., 2009). The same blood pool used for this experiment by Terry et al. (2009) was also used for sFIDA, an approach developed by Bannach et al. (2012). sFIDA exploits the strong tendency of PrP TSE to form large aggregates. In this assay, the aggregates were partially purified from plasma by precipitation with phosphotungstic acid, captured on a glass surface by a covalently bound antibody, labelled with a fluorescent antibody and then detected by confocal laser scanning at the single particle level, at which the signal intensity is proportional to aggregate size. Although the signals from scrapie-positive sheep were of variable intensity, sFIDA showed reproducibility in discriminating positive and negative plasma samples. Consistent with the results obtained by Terry et al. (2009), this assay displayed a 55–60 % sensitivity, whereas it detected PrP TSE aggregates in the plasma of nearly 60 % of the clinically affected sheep that were naturally infected with scrapie, with no false positives. sFIDA unequivocally demonstrated that disease-associated PrP aggregates are not only associated with cells in the blood, but are also present as soluble molecules in plasma. When this test was applied to plasma from BSE-affected cattle, it was not able to detect PrP TSE aggregates in either clinical or preclinical samples, suggesting that, in these cattle, infection may be limited to the central nervous system (Bannach et al., 2013). sFIDA has been proposed by its developers for use as a complementary or confirmatory blood test.

These two assays are based on capture of PrP TSE by an appropriate ligand before immunodetection. They detected PrP TSE in plasma anduffy coat from sheep and cattle infected with scrapie and BSE, respectively, with 55–60 % sensitivity and 100 % specificity. The main disadvantage of these two assays is their insufficient sensitivity for the detection of symptomatic and asymptomatic TSE-infected individuals.

**Flow cytometry**

This latter finding was in strong contrast to that of an earlier study in which PrP aggregates were detected in the serum of BSE-affected cattle during the clinical stage of the disease by flow cytometry (Triessmann et al., 2005). This method exploited the kinetic differences between seeded and unseeded polymerization of PrP monomers, and was able to detect synthetic PrP aggregates at concentrations lower than 10^{-8} nM (0.24 fg ml^{-1}) with 100 % specificity and sensitivity.

**Multimer detection system (MDS)**

The PrP-aggregate detection approach adopted by An et al. (2010) included an ELISA-based MDS for PrP TSE. Their assay consists of a modified sandwich ELISA in which only multimers of PrP TSE are specifically and selectively identified by antibodies after capture, because at least one copy of the same epitope remains exposed on the surface of the multimer (An et al., 2010). Using this MDS approach, multimers of PrP were specifically detected in the plasma of terminally ill, 263K-infected hamsters, but not in the plasma of uninfected hamsters. However, a major limitation of this assay is that its success depends on the absence of multimeric PrP in normal samples and the exposure of an epitope on PrP TSE, a prerequisite that could not be satisfied under different experimental conditions or with other animal species or prion strains. Moreover, due to the underlying principle of detection, this method has a lower sensitivity (approximately 10 ng, equivalent to approximately 3 nM protease-resistant mouse PrP from infected brain homogenate, spiked into mouse plasma) compared with the other assays described in this review.

This test was based on the selective detection of multimers of PrP TSE. It detected such multimers in the plasma of hamsters infected with the 263K strain of scrapie. Its detection sensitivity is the lowest of all tests. Its main disadvantage is that, due to the principle behind PrP detection, it is very limited in its application.
Amorfix EP-vCJD

The tendency of PrP to aggregate has also been exploited by the developers of the Amorfix EP-vCJD blood screening assay, the first commercially available diagnostic immunoassay (Amorfix Life Sciences Press Release, 2008), which has been re-evaluated for feasibility by the Guntz laboratory (Guntz et al., 2010). Using this assay, Amorfix performed a blind test of 1000 human plasma samples that had been spiked with vCJD-infected or uninfected brain homogenate. Later, Guntz et al. (2010) were able to test more than 20,000 plasma samples from French donors. The test was performed with 1 ml of not yet leukoreduced plasma, and PrP aggregates were concentrated from plasma samples and disrupted by denaturation. The resulting monomers were detected by a sensitive and specific immunoassay based on a europium-fluorescent bead bound to the 3F4 antibody (aa 108–111) and a magnetic bead bound to the 6H4 antibody (aa 144–152). In this assay, europium fluorescence intensity is directly proportional to the concentration of PrP\textsuperscript{TSE} in the sample.

This test was highly efficient, not only with fresh samples, but also with samples frozen at −30 °C and when it was performed with 12-month-old kits, which demonstrated its excellent shelf life and stability. Moreover, the technique can be completed very quickly (in approximately 3.5 h) and does not require a PK-digestion step; however, it does require epitope protection to selectively identify PrP aggregates. Furthermore, it showed 100% sensitivity and 97.6% specificity upon initial testing, which increased to 100% on repeat reactive testing. According to Amorfix, this kit allowed detection of as little as 4 fg per well of recombinant human prion protein and a 1:100,000 dilution of vCJD brain homogenate. Based on comparative bioassays and the blood titres in animal models (Cervenakova et al., 2003; Gregori et al., 2004; Minor, 2004; Herzog et al., 2004), this detection limit was tenfold more sensitive than the minimum required by Blood Transfusion Services in the UK (European Commission DG Health and Consumer Directorate, 2012). However, this test was not suitable for routine testing of blood donations, as it did not meet traceability requirements. The traceability could be improved by using automated systems to distribute and identify samples. Moreover, because no samples from individuals clinically affected with vCJD were tested, proof that this test can detect endogenous prions is still lacking, and a confirmatory assay is required to distinguish a true positive from a non-specific reaction, because no samples were positive in all three repeat experiments performed.

This assay is based on the concentration and disruption of PrP\textsuperscript{TSE} aggregates and immunofluorescence detection of the monomers. It displayed 100% sensitivity and specificity. It detected PrP\textsuperscript{vCJD} in human plasma spiked with human vCJD-infected brain homogenate. However, it has not yet been applied to endogenously infected samples. It is a multistep assay, and it did not meet traceability requirements.

Conformation-based approaches

Misfolded protein diagnostic (MPD) assay

Another approach to TSE diagnosis exploits the conformational differences between PrP\textsuperscript{C} and PrP\textsuperscript{TSE} by using the MPD assay and the dissociation-enhanced fluoroimmunoassay (DELFIA) method. The MPD assay is based on conformational amplification of PrP\textsuperscript{TSE}, which can be achieved by using a conformationally sensitive pyrene-labelled peptide (33-H1) from the N-terminal region of PrP, which contains conserved amino acid sequences (Pronucleon ligands) that, in the presence of PrP\textsuperscript{TSE}, are able to change into a β-sheet structure (Pan et al., 2007). The signal for the presence of PrP\textsuperscript{TSE} is amplified upon propagation of the conformational change to other peptide molecules. In an earlier study, it was shown that these peptides could detect PrP\textsuperscript{TSE} in the brain of hamsters infected with the 263K strain of scrapie during the preclinical and clinical stages of the disease. In the study by Pan et al. (2007), PrP\textsuperscript{TSE} was specifically detected with a threshold of sensitivity of about 1 ID microlitre\textsuperscript{−1} in the plasma of mice infected with the Fukuoka-1 strain of Gerstmann-Sträussler-Scheinker (GSS), squirrel monkeys infected with human sCJD, and humans affected with sCJD, and in the serum of sheep with endemic scrapie. However, using synthetic substrates to induce maximal excimer formation, where the peptides undergo a detectable conformational change induced by the presence of PrP\textsuperscript{TSE}, appears to be critical to the success of this technique. Moreover, there is a strong possibility of non-specific amplification due to conformational changes that are induced by other β-sheet proteins or high-abundance proteins present in blood.

The MPD assay is based on conformational amplification of PrP\textsuperscript{TSE} by using a synthetic peptide. It detected PrP\textsuperscript{TSE} in plasma and serum from mice, sheep, monkeys and humans infected with endemic scrapie, GSS or sCJD. The fact that this assay relies on a reactive synthetic peptide for both amplification and specificity is its major disadvantage, and it is also time consuming.

DELFIA

DELFIA is a sensitive immunoassay with time-resolved fluorescence detection, in which an anti-PrP antibody is labelled with lanthanide chelates, most commonly europium, which emit stable fluorescent signals. DELFIA was able to distinguish CJD-infected from uninfected human plasma samples, without PK digestion. Additionally, it detected PrPres in PK-digested human plasma samples spiked with hamster or human recombinant PrP, or with brain homogenate from patients with CJD (Völk et al., 2001). However, it was not able to detect PrPres in the plasma of patients with sCJD.

Other approaches

Other approaches used for the diagnosis of TSEs include acidic SDS precipitation and immunocapillary electrophoresis assays. The acidic SDS precipitation method was
A routine blood test for prion diseases

Tests based on detection of biomarkers other than PrP

Tests based on detection of downregulated or upregulated proteins

SELDI-TOF MS. Recently, some researchers have attempted to identify non-prion protein biomarkers for TSEs (Table 1). Using a SELDI-TOF MS technique to analyse the serum of scrapie-infected Syrian hamsters and sheep, Batxelli-Molina et al. (2010) identified some potential biomarkers whose expression differentially and significantly changed during the course of the disease. SELDI-TOF MS analysis of the profiles of proteins obtained from serum samples of sheep with scrapie (in the early and late phase) and normal controls identified 15 different peaks that were mostly expressed in the infected samples compared to the normal samples. Because none of the 15 peaks was specific enough to be used in isolation as a biomarker for either the early or late phase of the disease, using a statistical program (mROC), the authors determined that a combination of four differentially expressed peaks in either the early or late phase of the disease is more reliable as a biomarker of scrapie than any of the individual peaks. The detection sensitivity and specificity for the combined biomarkers were 87.3 % and 90.8 %, respectively, in the early phase and 69.8 % and 76.9 % respectively, in the late phase.

The masses of three of these 15 candidate biomarkers have also been detected in the protein profile of serum collected from 263K-infected hamsters at different time points during disease progression, but not in the profile from controls. This result indicated that such biomarkers of scrapie are not exclusive to sheep, but can be differentially expressed in other scrapie-infected species with respect to the uninfected controls. One of the three potential biomarkers that are common between sheep and hamster, that has been identified by both immunoblotting and liquid chromatography (LC)-MS/MS techniques, is a major fragment (15.7 kDa) of the transthyretin monomer. Although this marker displays some variation in expression that may be due to differences in sample handling and in the animal model used (Batxelli-Molina et al., 2010), it was shown to hold particular promise for use in early and late TSE diagnosis. Transthyretin has already been identified as a molecule associated with neurological disorders, such as multiple sclerosis, amyloid polyneuropathy, and TSEs, such as CJD, where it has been found in patient CSF (Batxelli-Molina et al., 2010). However, the authors concluded that complementary studies are needed to confirm the relevance of these proteins as TSE biomarkers.

This assay is based on a proteomic analysis by MS. It identified some potential biomarkers, for example transthyretin, which was shown to hold particular promise for use in early and late TSE diagnosis, in serum from sheep and hamsters infected with scrapie. The detection sensitivity in serum from sheep for combined biomarkers of the late and early phase of the disease was 69.8 % and 87.3 %, respectively, and the specificity was 76.9 % and 90.8 %, respectively. The main disadvantage of this assay is the difficulty in finding single, specific, reliable biomarkers.

ELISA. Another non-prion protein proposed as a biomarker is the heart-type fatty acid-binding protein (H-FABP), which was initially detected with 100 % sensitivity and specificity in the CSF of nine CJD- and nvCJD-affected patients and then, for the first time, with 71.4 % sensitivity and 100 % specificity in the plasma of a different group of CJD-affected patients. H-FABP levels were shown by ELISA to be significantly increased in both the CSF and plasma of CJD patients compared to the levels in control individuals (Guillaume et al., 2003).

Tests based on the detection of changes in the glycosylation of PrP and total plasma proteins

Since glycoproteins constitute one of the major subproteomes of blood (Madera et al., 2007), and because aberrant alterations in protein glycosylation are associated with some neurodegenerative disease states such as CJD and Alzheimer’s disease (AD) (Saéz-Valero et al., 2003; Silveyra et al., 2006), lectin-based diagnostic blood tests, which are able to detect glycosylation differences in the glycoproteins from TSE-infected and uninfected blood samples, have been evaluated by some research groups (Table 1). Lectins are a group of proteins that specifically bind to or cross-link carbohydrates on the surface of proteins, which makes it possible to quantify or identify different sugar types (Debray et al., 1981).

MS-based proteomics. One proposed lectin-based assay was developed by Wei et al. (2011), who used sequential enrichment of glycoproteins by a multi-lectin column, containing both concanavalin A (Con A) and wheatgerm agglutinin (WGA), and multidimensional LC to separate tryptic peptides prior to MS analysis. This group identified, for the first time, in control and RML-infected mouse plasma collected at different time points after infection, some low-abundance proteins, such as serum amyloid P-component (SAP) and apolipoprotein E (ApoE) (Wei et al., 2011). ApoE is the major component of low-density lipoproteins. It is associated with age-related risk for AD.
and plays a critical role in amyloid-β homeostasis. ApoE is also involved in TSE progression, and elevated concentrations have been detected in the brains of scrapie-infected mice and in association with some PrP aggregates (Diedrich et al., 1991). An earlier study showed higher concentrations of ApoE in the CSF of patients with vCJD than in the CSF of patients with sCJD (Choe et al., 2002). In the study by Wei et al. (2011), ApoE was shown, for the first time, to be upregulated in prion-infected mouse plasma at all three time points at which plasma was collected after infection.

The plasma levels of SAP, a 204-residue secreted glycoprotein with a single glycosylation site at position 51, was significantly changed during disease progression at two of the three time points tested. SAP glycosylation was significantly higher in the plasma of infected mice than in that of uninfected mice (3.4-fold) at 108 days post-infection.

Western blot analysis with an anti-SAP antibody showed two bands, at 26 and 30 kDa. At 108 days post-infection, the level of the 30 kDa SAP fragment was higher in infected samples than in normal samples, whereas the level of the 26 kDa SAP fragment remained unchanged. Deglycosylation with PNGase F confirmed that the 30 kDa band was the glycosylated form of SAP, the 26 kDa was the unglycosylated form, and that the elevation of SAP previously observed in MS analysis of infected samples could be fully attributed to the increase in its glycosylation. It has been reported that SAP co-localizes with neurofibrillary pathology in various neurodegenerative diseases, including AD, CJD, Parkinson’s disease and Lewy body disorders (Wei et al., 2011). However, because of the multistep sample processing, dynamic range of detection, random precursor selection in the MS/MS analysis, and the fact that many differentially regulated proteins are present at very low levels, this method did not allow reproducible detection.

This assay is based on the sequential enrichment of glycoproteins with a multi-lectin column, separation by high-pH RP-LC, and analysis by MS. It identified SAP and ApoE as potential biomarkers in the plasma of mice infected with RML. However, it is a multistep process and is not reproducible.

**Lectin-based ELISA and blotting.** Using ELISA and Western blot techniques, a panel of seven lectins revealed substantial glycosylation changes in plasma total proteins between 263K-infected and uninfected hamsters (Abdel-Haq, 2012). The glycosylation of some proteins differed after exposure to the prion agent. Lectin blots revealed that the glycosylation of glycoproteins at approximately 150, 116, 60, 50, 40 and 24 kDa was consistently altered in the plasma of infected animals, compared with that in the plasma of uninfected animals (Fig. 1). In particular, the 60 kDa glycoprotein, which is present as a double band, showed increased glycosylation of the upper band and the disappearance of the lower band in infected plasma compared with the normal plasma. This latter change was observed more clearly with WGA, and less so with *Ricinus communis* agglutinin (RCA). The glycoprotein at approximately 40 kDa, which may be the most affected, showed alterations in the N-acetylglucosamine and N-acetylgalactosamine sugars that are recognized by WGA and RCA, respectively. Additional immunological analysis capable of identifying some of these proteins with greater precision, particularly the 40 kDa protein, may also facilitate the identification of specific biomarkers for the diagnosis of scrapie and other prion diseases.

Additionally, PrPC and PrP TSE obtained from equal amounts of uninfected and infected plasma respectively, collected at different time points after infection, were captured on ELISA plates using the 3F4 mAb and detected by lectin staining. This experiment showed altered binding reactivity with PrP TSE compared with PrP C (Fig. 2). Among the seven lectins, the binding reactivity of Dolichos biflorus agglutinin (DBA), which specifically recognizes the α-N-acetylgalactosamine sugar, to PrP TSE was markedly and significantly (P ≤ 0.01) lower in infected animals than in uninfected animals. This was not only seen at 40 days post-infection (46.0%), but even as early as 18 days post-infection, reaching a 72.6% reduction (Fig. 2). Compared with the result obtained with brain total proteins, the binding activity of DBA was shown to be the most altered, whether toward glycoproteins present in the blood or the brain, suggesting that detection of this alteration in binding activity may be a reliable marker of the disease.
In this investigation, it was demonstrated that altered glycosylation occurs at an early stage of the disease, confirming its presumed involvement in pathogenesis. At the molecular level, conversion of PrPC to PrPSc may occur after the glycosylation of PrPC is altered. At the clinical level, systematic dysfunction (reflected by the typical clinical signs of TSEs) may also occur after alteration of the glycosylation of functional glycoproteins, such as the acetylcholinesterase glycoprotein. Altered glycosylation of acetylcholinesterase reduces its enzymic activity, which is followed by a progressive reduction in the function of the cholinergic system, resulting in dementia (Silveyra et al., 2006). The identification and characterization of the altered glycosylation of proteins may not only provide validated and reliable biomarkers for disease onset, but may also provide new targets for therapy and disease progression monitoring.

This assay is based on differential lectin binding to glycoproteins from infected and uninfected samples. It identified two glycoproteins, at approximately 40 and 60 kDa, as potential biomarkers in plasma of hamsters infected with the 263K strain of scrapie. It also detected PrPTSE after capture with an anti-PrP mAb, indicating the higher sensitivity of detection with lectins compared to that with antibodies. It suggested that detection of specific alterations in the binding activity of some lectins, such as DBA, might be a potential marker of the disease. It was shown to be not only diagnostic, but also useful for understanding pathogenesis. However, it is a multistep, time-consuming process. Its main disadvantage is its dependence on the quality of the collected samples.

**Discussion**

**Analysis and perspectives**

Given that a routine blood test should be simple, rapid, feasible, reliable, reproducible, highly specific and sensitive, inexpensive and non-invasive with a wide spectrum of applications, it is clear that none of the blood tests reviewed here satisfy all of these criteria. In particular, most of the tests are not sufficiently sensitive and/or their application in humans, sheep, cattle or preclinical samples has not yet been investigated. Moreover, none of these tests were sufficient alone; some of them required a confirmatory test, while others were proposed as confirmatory tests.

Although, a routine blood test has to be very quick, and completed with a few simple steps by any authorized technician, many of the reviewed tests are too time consuming, and some even require several days, multiple steps and special technical skills; therefore, they are not sufficiently reproducible. This limitation should be highlighted because it is particularly true for the PMCA method. PMCA is a technique that has spread worldwide and is frequently used to study prions, especially in the diagnostic field. The fact that the main drawback for some of these tests, including the PMCA method, is the high likelihood of generating false positives is in contrast to the reliability requisite of a routine test, which raises ethical, moral and legal issues. This is because such tests could potentially produce false alarms that would cause the diagnosed individuals and their relatives psychological and physical stress, as a reaction to the impact of such a diagnosis.

Another critical aspect is that some tests, which displayed a sensitivity and specificity very close or equal to 100 %, have been determined by analysing a very limited number of samples. Considering their application as routine blood tests based on this mediocre determination may pose a risk of frequent false alarms, raising the above-mentioned
ethical issues. Instead, the sensitivity and specificity of a candidate routine blood test should first be validated by using a larger number of samples, and then with actual samples (such as blood donations) through the use of a confirmatory test.

Some of the assays reviewed here, particularly the solid-state matrix assays (Lourenco et al., 2006; Edgeworth et al., 2011; Lacroux et al., 2014), demonstrated that it is possible to detect PrPTSE in the blood of symptomatic vCJD patients. This is a marked advance in the diagnosis of prion diseases, indicating that a blood test is possible, although not yet suitable, for routine use. These tests displayed low sensitivity, which appears to be the main obstacle to their routine employment. However, based on the fact that PrPTSE from vCJD-infected brain homogenate spiked into whole blood can be detected with good sensitivity at dilution of $10^{-10}$, hypothetically, it should be possible to detect prions in the blood of vCJD patients at the preclinical stage of the disease (Edgeworth et al., 2011). Moreover, given that other blood tests based on different principles showed similar behaviour, but that not all blood samples obtained from clinically affected subjects tested positive for PrPTSE (Terry et al., 2009; Bannach et al., 2012; Jackson et al., 2014; Lacroux et al., 2014), the sensitivity of the tests is unlikely to be the only factor responsible for the failed detection of PrPTSE. Rather, it may be related to the variable presence of factors intrinsic and extrinsic to blood that differentially affect the concentration and/or detection of PrPTSE in blood as compared to other tissues. This supposition is supported by the repeated confirmation that blood PrPTSE probably differs from brain PrPTSE in its conformation and aggregation properties (Bannach et al., 2012; Lacroux et al., 2012; reviewed by Lukan et al., 2013). It is also supported by the fact that PrPTSE present in blood is both associated with cellular components and in a cell-free state (Table 1), implying that, due dynamic equilibrium, the ratio of free to associated PrPTSE is continuously subject to change during the disease period. Consequently, the detection systems could detect concentrations of PrPTSE in blood that differ considerably among infected individuals, or in some individuals PrPTSE could even seem absent (Terry et al., 2009; Bannach et al., 2012; Jackson et al., 2014; Lacroux et al., 2014).

Potential limiting factors

Besides the well-known limiting factors associated with PrP in blood in comparison to that in brain, including the possible higher PK-sensitivity of PrPTSE (Brown, 2001b; Yakovleva et al., 2004), the higher ratio of PrP$^C$ to PrP$^Sc$ (Yakovleva et al., 2004) and the inhibitory action of high-abundance proteins (Trieschmann et al., 2005; Ernoult et al., 2010), there are other potential limiting factors that have not yet been considered, although their impact on the detection of PrPTSE and other proteins might be important.

1. The predominant presence of the aggregated form of PrP in the blood (An et al., 2010; Bannach et al., 2012) may mask its actual concentration, which would make it appear to be much lower than it is, due to the variability of the ratio of free to aggregated PrP$^TSE$.

2. The non-specific binding of several plasma proteins, including PrP$^Sc$ and the infectious agent, to fibrin clots which can often be made from plasma samples in vitro (Talens et al., 2012).

3. The entrapment of two important protease inhibitors, α2-macroglobulin and α1-antitrypsin, into fibrin clots (Talens et al., 2012) may increase the spontaneous digestion of PK-sensitive PrP$^Sc$, reducing its effective concentration in plasma samples.

4. Direct transfer of models developed for brain tissue and animals without proper adaptation to blood and humans, respectively, may affect the estimates of PrP levels in blood.

These factors, which are closely related to the unique properties of blood PrPTSE and individual differences in metabolism, which randomly modulate the concentration of detectable PrPTSE in blood, would consequently reduce the reliability of a blood test performed at a single time point during infection.

Future directions

This study emphasizes the need to not only increase test sensitivity, but also to adopt other strategies aimed at identifying and overcoming the limitations that have delayed the development of a routine blood test for prion diseases. Moreover, it may also provide a stepping stone for the development of diagnostic assays for other amyloid-related diseases.

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