EVALUATION CRITERIA FOR ANTE MORTEM DIAGNOSTIC TESTS FOR SUBCLINICAL vCJD

ISSUE

1. The UK blood services are preparing a systematic process to evaluate rapid ante mortem diagnostic tests for subclinical vCJD. The Department of Health (DH) has requested SEAC advice on general principles to consider for evaluation of such tests, which could be used by the blood services to develop an evaluation strategy. Ante mortem diagnostic tests for subclinical vCJD could also be used to inform on the prevalence of vCJD in the population and be used in the analysis of potential therapies.

BACKGROUND

2. Three cases of probable human transmission of prion infection from a blood donor who later developed vCJD have been reported, providing compelling evidence that vCJD infection can be transmitted via blood transfusion[1,2,3]. Blood donor exclusion criteria and other precautionary safety measures have been introduced since 1998 to reduce the number of potential iatrogenic cases of vCJD. However, the development of an accurate and sensitive ante mortem blood test to identify asymptomatic individuals infected with vCJD could substantially reduce the potential for transmission of vCJD via blood transfusion and other medical interventions. It could be clinically valuable in confirming the diagnosis of symptomatic cases and monitoring the effect of potential therapies. In addition, such a test could provide an important tool to ascertain better the prevalence of vCJD infections.

3. There are ethical and practical issues associated with ante mortem testing for subclinical vCJD. These have been discussed and comparisons made with testing for other blood borne diseases in Duncan *et al*[4] (Annex 1). Ethical

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[1] Peden *et al.* (2004) Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 364, 527-529.

[2] Llewelyn *et al.* (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion *Lancet*. 363, 411-412.

[3] http://www.hpa.org.uk/hpa/news/articles/press_releases/2006/060209_cjd.htm

[4] Duncan *et al.*(2005) Ethical considerations in presymptomatic testing for variant CJD. *J.Med Ethics* 31, 625-30

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considerations of ante mortem testing for subclinical vCJD have also recently been considered in detail by the Health Protection Agency. A report on this consideration is expected to be released in Autumn 2006 as well as a wider consultation of the recommendations. A report of the consultation is expected in spring 2007.

4. There are a number of rapid post mortem tests validated by the European Food Safety Authority (EFSA) for transmissible spongiform encephalopathies (TSEs) in animals[5]. A number of commercial organisations have recently claimed to have developed ante mortem tests that can be used on blood or other easily accessible biological material for the identification of asymptomatic individuals infected with vCJD. However, in the absence of a defined standardised system to evaluate and compare the effectiveness of these diagnostic tests, none has undergone formal independent evaluation and validation.

Previous scientific committee considerations

5. SEAC has not discussed the evaluation criteria for diagnostic tests for subclicial vCJD. However, this issue has been discussed in recent reports from the World Health Organisation (WHO)[6] and the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR)[7]. SEAC provided a response to the SCENIHR report consultation in February 2006. This issue has also been considered by a number of advisory groups to DH and an overview will be provided at SEAC 94.

WHO report

6. The WHO report states that:

The development of reliable diagnostic procedures to detect asymptomatic subjects during the long periods of incubation of CJD and vCJD is of vital importance. However, test methods must be appropriately validated, and validation requires that appropriate blood reference materials be developed, characterized and made available both to qualified test developers and to regulatory authorities.

[5] Raeber & Oesch (2006). Diagnostics for TSE agents. *Dev.Biol.* 123:313-23 and EFSA scientific reports on the evaluation of BSE/TSE tests. http://www.efsa.europa.eu/en/science/tse_assessments/bse_tse.html

[6] World Health Organisation (2006) Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies. http://www.who.int./bloodproducts/TSEREPORT-LoRes.pdf

[7] Scientific Committee on Emerging and Newly Identified Health Risks (2006) Opinion on the safety of human-derived products with regard to vCJD. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/scenihr_cons_02_en.htm

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Attempts to develop, optimize and validate tests to detect infectivity in blood in experimental and natural TSEs, including BSE in ruminants and vCJD in humans, are now in progress. These very important efforts should be encouraged and supported, both financially and by providing test developers with TSE reference materials and panels of replicate coded and randomized samples, including reference materials of human origin when available.

SCENIHR report

7. In considering the available data on ante mortem tests for subclinical vCJD, SCENIHR concluded that:

   Important advances in test methodologies for prion detection have been made in recent years, and the application of these advances in the diagnosis of vCJD has, in particular, been fruitful. However, no diagnostic system has yet emerged with the high level of sensitivity and specificity required for routine screening of blood or urine. It is essential that confirmatory assays are available, and all ethical implications are considered and carefully taken into account before implementing testing. Independent validation of any new methodology should be mandatory prior to implementation, and it is recommended to adopt the EU procedure used for the BSE testing. For validation carefully controlled vCJD reference materials should be used. However, the availability of blood from individuals at increased risk of vCJD or diagnosed with vCJD is very limited. Therefore, ethical collection of such valuable material should be considered a priority. Special strategies are then needed to evaluate potential blood tests in order to conserve material. Collection of urine should be considered which could be tested when more sensitive tests for abnormal prion protein suitable for testing urine become available. The issue of false positives needs careful consideration, as even minute percentages of these may actually involve a large number of individuals. The ethical issues of informing an individual of test results, without providing any certainty as to the likelihood of progression to clinical disease, should be considered seriously.

Diagnostic Tests

8. Due to commercial sensitivities, data on prototype rapid ante mortem tests for subclinical vCJD that have been developed are limited. However, the WHO and SCENIHR reports provide overviews of some of the most well developed prototypes (Annex 2). Generally, these assays detect abnormal prion protein (PrP^{Sc}) which is present in a biological fluid (blood or urine) as a marker of vCJD infection utilising:

   (i) the preferential binding of PrP^{Sc} to a chemically synthesised ligand

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(ii) the preferential binding of an antibody to an epitope of PrP^{Sc}
(iii) a combination of (i) and (ii)
(iv) the amplification of PrP^{Sc} using protein misfolding cyclic amplification

9. It is assumed that a good correlation exists between the presence of PrP^{Sc} in the biological material taken from an individual and vCJD infection. Some of the tests outlined could lend themselves to quantification of PrP^{Sc} and, given an assumption there is a good correlation between the concentration of PrP^{Sc} and infectivity levels, may be able to give an indication of the infectiousness of an individual. However, the uncertainties around the relationship between PrP^{Sc} concentrations and infectivity levels may preclude, or at least make such an assessment very difficult.

10. None of the prototype tests has a CE marking[8]. The regulatory framework that applies to diagnostic tests intended for market is outlined below.

Regulatory framework[9]

11. The In Vitro Diagnostic Medical Devices (IVD) Directive 98/79/EC is intended to ensure that IVDs do not compromise the health and safety of patients, users and third parties and attains the purpose and performance levels attributed to them by their manufacturer. The IVD Directive lists various essential requirements with which IVDs must comply before being placed on the market/put into service.

12. The IVD Directive groups IVDs into four categories. These categories are, in order of increasing perceived risk:

- general IVDs: all IVDs other than those covered by Annex II and IVDs for self-testing;
- IVDs for self-testing: a device intended by the manufacturer to be able to be used by lay persons in a home environment, excluding self-test devices covered in Annex II;
- IVDs in Annex II List B of the Directive: which, amongst others, includes reagents and products for rubella, toxoplasmosis and phenylketonuria as well as devices for self testing for blood sugar;
- IVDs in Annex II List A of the Directive: which includes reagents and products for human immunodeficiency virus I and II, hepatitis B, C and D.

[8] CE (Conformité Européene) mark is a declaration by the manufacturer that a product meets all the necessary requirements of the relevant EU legislation. http://www.ce-marking.org/directive-9879ec-IVD-MD.html
[9] The Medicines and Healthcare products Regulatory Agency has published full guidance on the In Vitro Diagnostic Medical Devices Directive in their Guidance Note 19 from which much of the following information has been taken.

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13. At present vCJD assays would be regulated under the general IVD category. They could be moved to Annex II list A at the request of Member States or the European Commission under Article 14 of the IVD Directive.

14. For products currently in List A of Annex II, conformity assessment may involve the use of "Common Technical Specifications" (CTS)[10] to establish performance evaluation and re-evaluation criteria, batch release criteria, reference methods and reference materials. CTS are drawn up by an Expert Panel convened by the Commission rather than by the Standards Bodies. Manufacturers are expected to comply with the CTS. If they have justified reasons for not doing so they must adopt solutions that they can prove are at least equivalent to the CTS in terms of the above criteria. As with harmonised standards, Member States must presume compliance with the essential requirements in respect of devices designed and manufactured in conformity with common technical specifications.

15. There are however, practical problems in drawing up a meaningful CTS for diagnostic tests for subclinical vCJD, for example, suitable reference materials for performance evaluation and batch verification will need to be sourced.

16. The Medicines and Healthcare products Regulatory Agency is currently considering a request to make a duly substantiated request to the Commission to have diagnostic tests for vCJD placed in Annex II List A. If the Commission and other Member states agree then they may convene an Expert Panel of impartial national experts from Member States to draw up a CTS. If the European Commission decides to go ahead, then they may ask an Expert Panel to draw up a CTS. Any such panel would need to be drawn from impartial experts from Member States.

Reason for request for advice

17. It is expected that some commercial companies will soon have assays suitable for evaluation as screens of blood and tissue donations for the presence of PrP^{Sc}. In order to provide a consistent approach to how these assays might be viewed and assessed from an operational viewpoint ahead of any CTS produced by the European Commission. DH and the blood services, have requested the formation of an expert group to inform companies of the criteria that they need to meet before a proposed test is analysed.

[10] Commission Decision of 7 May 2002 on common technical specifications for *in vitro* diagnostic medical devices (notified under document C (2002) 1344).

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Important characteristics of a diagnostic test

18. Considerations in relation to evaluation and validation of ante mortem tests for humans and animals and the validation of post mortem tests by the European Union are discussed further in the WHO report (Annex 3). Three important characteristics of a rapid diagnostic test would appear to be:

- reproducibility (the agreement between results of tests on the same samples tested in different laboratories)

- sensitivity (the probability that a truly infected individual will test positive when the test is applied)

- specificity (the probability that a truly non-infected individual will test negative when the test is applied.)

19. The sensitivity and specificity of a test govern the rate of false negative and false positive results, respectively. An analysis by DH of the required specificity and sensitivity of a diagnostic test for the UK blood services is given at Annex 4. The major implication of this analysis is that any test must be of very high specificity while the sensitivity and specificity could be lower for other purposes such as diagnostic testing prior to high-risk surgery. A scheme that involves a screening test, followed by a confirmatory test, for reactive samples (samples that test positive in the screening test) may provide a more practicable solution to address the specificity required for blood testing as the specificity required for the screening and confirmatory tests is not as great compared with that required for a single test. The use of such a system was a strong recommendation from an expert subgroup of the Committee on Microbiological Safety of Blood and Tissues that considered this issue[11].

20. The reproducibility, sensitivity and specificity of a test could be assessed by (i) review of the test manufacturer's data on the performance of the test, (ii) repetition of the manufacturer's experiments by an independent laboratory and/or (iii) assessment of the performance of a test by one or more laboratories when applied to analysis of a panel of standard samples that have been well characterised in terms of their composition (reference samples). Although (i) and (ii) provide an indication of the performance of the test, (iii) will allow the performance of tests to be compared and provide assurance that a test is robust and produces consistent results when applied by different laboratories.

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[11] MSBT Subgroup on vCJD of 17th Feb 2003

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21. Reference samples have been developed by the National Institute for Biological Standards and Control (NIBSC) using brain homogenate from two sporadic CJD (sCJD) cases, one vCJD case and a normal brain[12]. However, these samples are intended for use in small quantities for calibration purposes. NIBSC has also prepared samples of two spleens and a brain from vCJD cases, a brain from a sCJD case and a normal brain. Dilutions of these materials have been spiked into human plasma for use as standards (Annex 5). A panel of samples from American blood donors is also in preparation by the UK blood services for use as uninfected control samples.

22. SEAC (SEAC 91, February 2006) has previously noted that blood spiked with TSE infected brain or spleen homogenate may not be representative of endogenous infectivity in blood[13]. Therefore, use of spiked samples of blood may not provide the necessary assurance about the performance of a test in the clinical situation. However, quantities of blood from vCJD cases for use as reference samples to evaluate tests are in very short supply. Furthermore, as this blood is from clinical cases, it may not provide a clear indication of the ability of a test to detect subclinical infections. The blood from vCJD cases could be diluted with blood from uninfected individuals to represent blood from asymptomatic individuals infected with vCJD. In addition, individuals defined as 'at risk of vCJD for public health purposes' could provide an additional source of blood samples with endogenous vCJD infectivity. SEAC previously suggested (SEAC 91, February 2006) *that blood from individuals considered 'at risk of vCJD' should be collected with ethical approval and patient consent*[14]. Use of samples from vCJD cases or from individuals considered 'at risk of vCJD' may provide an indication of the ability of a test to correctly identify infected individuals. However, the change in the levels of vCJD infectivity (and PrP^{Sc}) in blood through the incubation period of the disease is uncertain. Therefore, use of these samples in an evaluation may not provide a good indication of the ability of a test to detect infection from the point in the incubation period when blood becomes infectious.

23. At SEAC 92 (April 2006), the committee considered the available data on the change in infectivity in blood during the incubation period and concluded *that the available data show that blood is infectious during the preclinical stage of vCJD. Although the precise time in the incubation period of vCJD at which blood becomes infectious is unclear, data from animal models suggests it may be infectious from at least, if not before, the middle of the incubation period*[15]. Thus, ideally blood samples collected over the duration of the incubation period or at least from, if not before, the middle of the incubation period would be useful to

[12] Minor *et al.* (2004) Standards for the assay of CJD disease specimens. *J.Gen.Vir* 85, 1777-1784.
[13] SEAC paper 91/3
[14] SEAC 91 minutes paragraph 32.
[15] SEAC (2006) Position statement on TSE infectivity in blood. http://www.seac.gov.uk/statements/statement0806.htm

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assess the ability of a test to detect infection from the point in the incubation period when blood becomes infectious. Such samples could not be collected from humans but could be obtained from animal models. At SEAC 91 (February 2006), the committee considered and provided advice on the use of blood from a number of different animal models including rodents (wild type and transgenic), sheep and non-human primates infected with human or adapted human strains of TSEs for use in the evaluation of prion reduction filters (Annex 6). Some of this advice may be applicable to the preparation of reference samples to evaluate ante mortem tests for subclinical vCJD.

24. At SEAC 94 the committee will receive presentations on the scientific basis of rapid ante mortem tests for subclinical vCJD, the characteristics of tests required by the blood services, development of reference materials for evaluating tests for vCJD and general requirements for an effective diagnostic test for infectious blood borne diseases.

ADVICE SOUGHT FROM THE COMMITTEE

25. The committee is asked to:

- consider endorsing the independent evaluation of diagnostic tests prior to implementation.
- provide scientific advice on how best to establish the reproducibility, sensitivity and specificity for rapid ante mortem tests for subclinical vCJD and assess the ability of a test to detect infected individuals from the point in the incubation period when blood may be infectious.
- comment on the scientific rationale for using the presence of abnormal prion protein as a surrogate marker of infection in an individual.
- produce a position statement from the discussion.

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Extract from WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006). Annex 2 Summary of Scientific Presentations. Evaluation Of TSE Blood Transmission Risk Blood Screening Tests- page 38

and

Extract from Scientific Committee On Emerging And Newly Identified Health Risks (SCENIHR) Opinion on: The Safety of Human-derived Products with regard to Variant Creutzfeldt-Jakob Disease- Diagnostics. Section 3.3.5 Tests in Development for Blood.

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Extract from WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies

Evaluation Of TSE Blood Transmission Risk
Blood Screening Tests

C Orser, H Perron, B Phelps, C Soto, S Wilson, A Raeber

Several tests in development were described as having a demonstrated or potential capacity to detect PrP^{TSE} in blood. The first method relied on a misfolded protein diagnostic (MPD) assay using a conformationally responsive palindromic polypeptide ligand able to detect PrP^{TSE} directly without protease treatment (Tcherkasskaya, Sanders et al. 2003; Grosset, Moskowitz et al. 2005).

Preliminary results reported were limited to the symptomatic stage of disease and yielded positive tests in all of 52 scrapie-infected sheep (but none of 45 normal sheep), all of 43 scrapie-infected hamsters (none of 23 normal control hamsters), 36 of 40 BSE-infected cows (one of 40 normal cows), all of 14 humans with CJD (none of 53 normal humans), all of eight CJD-infected monkeys (none of four normal monkeys), and all of five CJD-infected mice. The limit of level of detection for PrP^{TSE} in plasma was estimated at 1 ID/ml.

The second method is a research prototype that detects proteinase K-resistant PrP^{TSE} in blood. The first step concentrates soluble oligomers of PrP^{TSE} in plasma (using aliquots of 10 µl for mice, 50 µl for humans and sheep and 500 µl for cattle) and then treats with proteinase K followed by precipitation with streptomycin and centrifugation; after denaturation the remaining PrP is detected with a sandwich ELISA that captures PrP^{TSE} aggregates with calix-arene molecules and detects them with a labeled anti-PrP antibody (Bencsik, Coleman et al. 2006; Moussa, Coleman et al. 2006). In preliminary studies, seven of ten plasma samples from CJD patients were reactive while samples from 500 healthy blood donors were negative. Spiked material did not reproduce results observed with naturally infected blood. No standardized blood materials were available to test. The third method uses peptides coated on magnetic beads as PrP^{TSE} specific binding reagents to bind PrP^{TSE} in plasma without proteinase K treatment. After dissociation from the beads, PrP^{TSE} is detected by sandwich ELISA. The sensitivity reported with human and sheep brain homogenates spiked in control plasma was 3 ID/ml (a one-million-fold dilution of 10% brain homogenate). Preliminary data indicated that samples from three of eight scrapie sheep tested were reactive (using 70 µl plasma in each test). The fourth method used the automated protein misfolding cyclic amplification (PMCA) technique (Castilla, Saa et al. 2005; Soto, Estrada et al. 2006), reported to amplify PrP^{TSE} at least 6600-fold. One round of PMCA was reported to increase sensitivity of detection 2500 fold when compared to titre by

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immunoblotting in an initial tissue suspension and after two rounds of PMCA (fresh normal tissue extract added to each new round) an amplification factor of 6.5 million was claimed. PrP^{TSE} was first detected in the blood buffy coat of 263K-scrapie-infected hamsters 20 days after intraperitoneal infection (three of six animals), and in 89% of clinically ill animals (16/18 animals 100 days post-infection) while blood samples from none of 12 uninfected control hamsters reacted. The technique has not yet been adapted to detect PrP^{TSE} of humans or sheep. The fifth method, relying on proprietary ligands coated on magnetic beads without PK treatment, uses samples of 225 µl of plasma. The protocol resembles that of the third method, including PrP^{TSE} capture, wash, elution, denaturation and final detection by ELISA. Spiked samples containing 1-10 mg vCJD spleen/ml of plasma (equivalent to 102-103 ID/ml) were detected in a blind study. Plasma from two scrapie sheep of three tested were reactive versus none of 26 samples from scrapie-negative control sheep. The last method presented used an antibody (IgM 15B3) specific for PrP^{TSE} (Korth, Stierli et al. 1997; Nazor, Kuhn et al. 2005) to capture and enrich it from serum or plasma (200-µl aliquots) and subsequent detection with a labelled secondary anti-PrP specific antibody. The assay uses either ELISA or fluorescence-activated cell sorting formats. PrP^{TSE} was detected in dilutions of scrapie sheep brain up to 1/66,000. Plasma samples all of six scrapie sheep were reported to be reactive versus no reactive samples from 16 control sheep. Samples from three of 12 BSE cattle were reactive versus no positive samples from ten cattle. So far, none of these candidate tests has been evaluated independently and no reference materials comprised of blood specimens from documented TSE-infected animals or humans are available to assist in the blinded objective comparison and convincing validation of these methods.

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Extract from SCIENTIFIC COMMITTEE ON EMERGING AND NEWLY IDENTIFIED HEALTH RISKS (SCENIHR) Opinion on The Safety of Human-derived Products with regard to Variant Creutzfeldt-Jakob Disease- Diagnostics

3.3.5 Tests in Development for Blood

The development of assays for TSEs based on blood samples has been a focus of activity for commercial and other groups for some time and is apparently making real progress (Brown 2005). Currently the most promising approaches aim to detect PrPSc. Few of the relevant studies have appeared in the peer reviewed literature because of commercial interests although some of the enabling technology has been published. The applications of the technology have been presented at meetings and in general the level of detail available is less than would be required for a scientific publication. It seems probable that in the very near future detection of infected individuals in the preclinical and subclinical phases through testing blood samples will be possible. For the purpose of this opinion it was decided to review the methods, bearing in mind that the list may be incomplete and the supporting information inadequate.

Early approaches to assays of very low amounts of PrPSc included immunocapillary electrophoresis (ICE) (Schmerr et al 1999) in which samples were digested with proteinase and residual PrP was detected by competition with a synthetic peptide for binding to a specific antibody. The key features were the concentration of the blood sample and resolution of the complexes by capillary electrophoresis. The method was technically complex and proved difficult to reproduce on human and chimpanzee samples (Cervenakova et al 2003). A modified method has been published, but is not yet validated (Yang at el 2005). SIFT (Screening for Intensely Fluorescent Targets) is based on the fact that because PrPSc aggregates, it presents a higher number of antibody binding sites than the unaggregated normal form, so that fluorescent intensity in immunoassays is more intense for PrPSc (Bieschke et al 2000). While this is a sensitive method in the detection of known infectious material such as brain samples, the current state of development is not known and it has not apparently been applied to blood. As already discussed above, CDI under the right conditions is very sensitive (Safar et al 1998, Safar et al 2005, Bellon et al 2003), and its suitability for the assay of blood is currently being explored.

A palindromic ligand has been developed by Adlyfe which converts from the alpha helical to beta sheet form in response to interactions with PrPSc. The transition is detected by fluorescence, and the method is sensitive (Grosset et al 2005). It has been reported to distinguish samples from infected and uninfected laboratory animals,

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sheep and cows (Pan et al 2004). A ligand (Seprion) has been developed by Microsens which is reported to bind specifically to PrPSc of any species and to distinguish blood from infected and uninfected sheep (Lane et al 2003). It has also been reported to distinguish blood from an iatrogenic case of CJD from controls (Wilson 2004). An antibody, 15B3, reported to be specific for PrPSc (Korth et al 1999) has been used in the development of methods for the detection of PrPSc in blood (Zwald et al 2004).

Finally, the cyclic amplification of PrPSc in vitro (PMCA) has been reported to be sufficiently sensitive to detect prions in blood from the preclinical phase (Castilla et al 2005b, Soto et al 2005).

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Extract from WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006).

Approach to validation of tests: strategies for development of reference materials.

M Turner

There are important issues for blood services and national regulatory authorities to consider in attempting to develop, validate and eventually implement tests that identify infected donors during the pre-clinical phase of a TSE—donors who might have infectious agent in blood.

(a) The biological and analytical performance properties of a candidate test must first be well characterized: when during the course of the incubation period the test becomes positive relative to the appearance of infectivity and, especially, when the amount of infectivity is sufficient to infect a recipient of a blood component. In order to improve the safety of the blood supply meaningfully, a test might require an extraordinarily high analytical sensitivity, because the presence of a single human intravenous infectious dose in a unit of blood implies that 1/500 of an intravenous dose might be available for detection in a 1-ml blood sample.

(b) Of special importance are the clinical performance characteristics of a candidate test: its clinical sensitivity, specificity and positive and negative predictive values; in healthy donors screened for the first time for evidence of a rare infection, even a highly specific test—if not perfect—is expected to generate far more false-positive than true-positive results, yielding a very poor positive predictive value. After positives have been screened out of a donor population, the performance of the test would become even worse, with an expected positive predictive value far less than 1%. Development of any TSE test of practical use for screening donors must be accompanied by development of a second confirmatory test based on a different methodological principle that is at least as specific as the candidate screening test.

(c) Evaluation of TSE tests should be conducted by a strategy announced in advance and acceptable to an independent national authority. For example, UK authorities have proposed the following general scheme for a candidate test after it receives the Council of Europe mark. The test should discriminate between samples of normal peripheral blood spiked with dilutions of normal human brain extracts and blood spiked with extracts from brain and spleen tissues of patients with confirmed TSEs, presented in both unblinded and blinded panels of samples. The test should discriminate between samples of peripheral blood from normal animals and animals infected with TSEs, also presented in both unblinded and blinded panels of samples. The sensitivity

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of the test should be evaluated by its discrimination between samples of peripheral blood from persons at negligible risk for a TSE (including both normal subjects and persons with other neurological diseases) and those of patients with diagnosed vCJD, samples presented in both unblended and blinded panels. The test might also be applied to blood samples from persons thought to be at high risk for vCJD and, possibly, for other TSEs, like familial CJD. Clinical specificity should be evaluated by testing a large number of samples from healthy donors (at least 10,000).

(d) The assay should be feasible for blood programs. The assay should be practicable, requiring a reasonably small volume of donor blood and having a short report time. The test should be operational, using available technical platforms and technical staff expertise. Blood programs and national authorities should consider carefully, in advance, the potential impact of screening donors for TSE—including the implications of notification for persons testing positive, their families and society, as well as on the blood supply.

D Matthews

Historically, tests used for surveillance of BSE or scrapie first arose from research programs; the tests were introduced after limited validation and their performance was evaluated only later, during the course of surveillance. The situation changed with the advent of rapid tests for BSE, but, for several reasons, the evaluation of tests presented major challenges. Although it began the process on its own, the European Commission subsequently sought advice from its Scientific Steering Committee and later from the European Food Safety Authority (EFSA). Standardized reference BSE materials were not prepared, except for brain samples from New Zealand cattle as negative controls. Access to BSE-positive brains was therefore opportunistic, depending on supplies from individual EU Member States, especially the UK. One significant concern was to supply the test manufacturers with slices of brain stem, as opposed to prepared suspensions, so as not to compromise the tests if their performance depended on special homogenization techniques and to ensure that evaluation accurately reflected real test conditions. So it was difficult to produce adequate supplies of appropriate standardized BSE brain reference materials. After accepting evidence for proof of principle, tests were first evaluated for sensitivity and specificity. In 1999 this required testing samples from 300 BSE-positive and 1000 BSE-negative samples. In 2001 those numbers were reduced to 48 positives and 152 negatives, but the tests were subsequently subjected to field trials involving 200 BSE-positive brain samples, at least 10,000 BSE-negative samples, and, ideally, 200 poor-quality BSE-negative samples—the last to ensure that test performance was not compromised by autolysis. This format was followed in a subsequent evaluation in 2004. Live-animal tests present a much greater challenge. Current EU guidelines recommend evaluating sensitivity by testing with at least 138 BSE-positive samples to show with 95% probability that the sensitivity is not less than 98% (258 samples for

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99% sensitivity). A round figure target of 200 samples from known BSE-positive animals was chosen, aiming to confirm that sensitivity of a test is not below 98.5%. Again, determination of acceptable specificity demands the testing of at least 10,000 samples from BSE-negative animals. Unfortunately, for most BSE suspect animals for which samples were available, delays up to 72 hours occurred between collection and aliquoting and freezing, which may affect test performance. Only after evaluating live-animal testing with samples from animals having signs of BSE is consideration given to testing animals in pre-clinical stages of incubation. A few such samples have been collected from experimentally infected cattle, but the conditions of sampling may not suit each test. Because animals in the DEFRA-sponsored studies were slaughtered sequentially, the number of animals from which a full time-course series was collected is relatively small; for samples collected from other animals, it is possible only to confirm that the animals either were exposed to BSE agent or were not, especially because some tests might detect positive cattle only early in the incubation period, when central nervous system tissues still test negative for BSE. To achieve statistical credibility it might therefore be necessary to infect more animals in order to generate a collection of samples during both the asymptomatic incubation period of BSE and overt illness, but the costs of projects of adequate size would probably be prohibitive.

Having introduced a scheme in principle for all tests in 2005, the OIE plans to begin a process of test evaluations in 2006. The aim is to list post-mortem tests "fit for purpose". The standards to be achieved for approval, and their relationship to existing approval processes, have still to be established. The Scientific Steering Committee—later EFSA—of the EU set a "benchmark" goal for postmortem BSE tests used in Europe of 100% sensitivity and specificity; several tests have performed well. Although antemortem blood-based tests for BSE would be desirable, it will be difficult to evaluate and establish acceptance criteria for tests that rely on the detection of abnormal forms of the prion protein or other potential markers in blood during pre-clinical disease. EFSA has published an opinion about strategies for evaluating candidate antemortem BSE tests, but suitable reference materials for characterizing such tests are not available. A special need is for control materials from cattle with neurological diseases other than TSEs. UK and German authorities have assembled some materials suitable for evaluating BSE tests, but they are available in quantities collection supported by commercial or government funding not currently available.

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Eglin R and Bennett P (2003). Blood Screening for vCJD: Implications of Test Results.

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Blood Screening for vCJD: Implications of Test Results

`Roger Eglin (NBS) and Peter Bennett (EOR4 of DH)

1st April 2003[16]

For MSBT Subgroup on vCJD (Action 7(1) of meeting on 17th Feb 2003)

Background

1. Although a blood screening test to detect vCJD is not yet available, it has been suggested that one may become so within a few years. Though there would doubtless be great pressure to use any test as soon as possible, the consequences would not all be beneficial – hence the need forward / contingency planning. The need to evaluate a test could mean that many months, perhaps several years, might separate its discovery from its routine application; and once applied it might put strains on blood procurement.

2. In many scenarios (as discussed below) few of the positive test results would be truly indicative of the disease. Bearing this in mind , key general issues include the following:

   - Effect on the blood supply of a test, through:
     - Donor fear. NBS research suggests that if a test is introduced some donors may stay away for fear of knowing the result (though with HIV, some have deliberately used the opportunity to get tested).
     - The potential need to discard (perhaps unnecessarily) substantial quantities of donated blood
   - Management of "positives". Under EU regulations, those whose blood could not be used would *have* to be told why. Especially if there is still no effective treatment or cure available for vCJD, the effect on donors' lives could be devastating. In addition, loss of future donations from such individuals could be substantial.

3. Though not considered here, one might also wish to allow for the impact of a screening test on other risk reduction measures. Introducing a test would decrease the marginal benefit of other steps to reduce vCJD risks. These may have other benefits (for example leucodepletion reduces the risk of CMV transmission), and would also help deal with imperfections in the test. But the point remains that measures may be left in place despite greatly reduced benefits, given the difficulty of reversing any safety measure once implemented.

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[16] Minor amendment to Table 1 made July 3rd 2003

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Sensitivity and specificity

4. For simplicity here, we consider a single-stage test for vCJD, rather than an initial screening test backed up by some confirmatory procedure. However the discussion can be extended as appropriate. Two qualities of any test are sensitivity and specificity.

   - Sensitivity is defined as the probability that a truly infected individual will test positive when the test is applied.
   - Specificity is the probability that a truly non-infected individual will test negative.

5. False positive (FP) results are the consequence of poor specificity. For example, these can occur due to group cross-reactions between antibodies to different organisms with similar epitopes.

6. False negative (FN) results are due to poor sensitivity. Possible causes may include: natural or induced tolerance to the antigens (persistent infection), improper timing of the test relative to the stage of infection, improper selection of test, non-specific inhibitors, or the test being insensitive (unable to detect minimal levels).

7. Ideally, a test should be both highly sensitive and highly specific. In practice, there may be some trade-off to be made between the two. When considering selection of tests, the consequences of inaccurate disease classification must be considered. If the cost of a FP is high, a highly specific test is required. If the cost of a FN is high, a highly sensitive test is required. In this context, FNs obviously pose a potential risk to recipients of blood. FPs pose a risk to the supply of blood (as well as casting a shadow over the lives of uninfected donors), as blood may be unnecessarily discarded.

8. The reliability of a test result is governed by the factors just discussed, combined with the prevalence of the disease (the proportion of individuals in a population infected at a given time). Specifically:

   - <u>Positive predictive value</u> (PPV) is defined as the probability of true infection, given a positive test result. This can be expressed be the percentage of positive results that are true positives.
   - <u>Negative predictive value</u> (NPV) is probability that an individual testing negative is truly negative and is most relevant when the intention is to rule out infection. It can be expressed as the percentage of negative results that are true negatives.

9. Clearly, high specificity increases the PPV, by reducing FPs. Highly sensitive testing increases the NPV, by reducing FNs. Nevertheless the role of prevalence is also crucial. In particular, if a disease is rare (low prevalence), then a very high specificity may be required to prevent the test having poor PPV. Otherwise most of the “positives” indicated by the test will be False Positives. As a simple example, consider an assay

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with specificity of 0.999. Suppose this is used to screen the (roughly) 2.5 million donations received each year for a disease with a true prevalence of 1 in 10,000. The number of False Positives indicated would then be 2,500 - ten times the number of true positives in the population.

Predictive values: scenarios for vCJD tests

10. The true prevalence of this disease is of course unknown. So it is helpful to consider a range of scenarios for prevalence, test specificity and sensitivity. This is done in Table 1 below. The first two columns provide alternative scenarios for prevalence of infective individuals (taking the population of England and Wales as approximately 50 million). For each of these scenarios, the following columns show alternative assumptions (0.9 or 0.99) for both sensitivity and specificity, then the number of donations (from 2.5 million annually) that would test positive or negative. Finally, the last two columns give the predictive values for these positive and negative results.

Table 1: Scenarios for vCJD prevalence and test sensitivity and specificity

vCJD scenario		**Screening Test**		**Test results (numbers)**		**Predictive values (%)**	
Number infective	**Prevalence**	**Sensitivity**	**Specificity**	**Positive**	**Negative**	**PPV**	**NPV**
500000	0.01	0.9	0.9	270,000	2,230,000	8.3333	99.8879
	(1/100)	0.9	0.99	47,250	2,452,750	47.619	99.8981
		0.99	0.9	272,250	2,227,750	9.0909	99.9889
		0.99	0.99	49,500	2,450,500	50.000	99.9898
50000	0.001	0.9	0.9	252,000	2,248,000	0.8929	99.9889
	(1/1,000)	0.9	0.99	27,225	2,472,775	8.2645	99.9899
		0.99	0.9	252,225	2,247,775	0.9813	99.9989
		0.99	0.99	27,450	2,472,550	9.0164	99.9990
5000	0.0001	0.9	0.9	250,200	2,249,800	0.0899	99.9999
	(1/10,000)	0.9	0.99	25,222	2,474,752	0.8921	99.9990
		0.99	0.9	250,222	2,249,777	0.0989	99.9999
		0.99	0.99	25,245	2,474,755	0.9804	99.9999
500	0.00001	0.9	0.9	250,020	2,249,980	0.0090	99.9999
	(1/100,000)	0.9	0.99	25,022	2,474,977	0.0899	99.9999

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		0.99	0.9	250,022	2,249,977	0.0099	100.0000
		0.99	0.99	25,042	2,474,975	0.0989	100.0000

11. It can be seen that while the predictive values of negative tests (NPVs) remain high, those for PPV never exceed 50%, and in most scenarios are very low. In other words, most positives are in fact false, with significant numbers of donations being discarded (and donors informed) unnecessarily.

12. Table 2 below extends the discussion by considering how further increases in sensitivity and specificity against a fixed prevalence of the disease (of 1 in 100) affect the Positive Predictive Value.

Table 2: Test Sensitivity and Specificity against fixed Disease Prevalence

Test		**PPV (%)**
Sensitivity	**Specificity**	
0.9	0.9	8.3
0.99	0.9	9.1
0.999	0.9	9.2
0.9	0.99	47.6
0.9	0.999	90.9
0.99	0.99	50.0
0.999	0.999	90.0

13. Summarising from both tables, it can be seen that:

- As the prevalence of infection decreases, so does the PPV of a given assay.
- At low prevalence of infection, the PPV of even a highly specific assay will be poor. But the NPV is typically high, even for a test of modest sensitivity.
- At a fixed prevalence, increasing the sensitivity has little effect on the PPV.
- At a fixed prevalence, increasing the specificity increases the PPV.

Implications

14. For vCJD it is expected that all, or at least the majority, of primary infections have taken place. So it may be reasonable to assume the prevalence of infection is fixed, at least to a first approximation. If prevalence is assumed to be low, it is essential that any assay selected for use as a screening test has as high a specificity as possible.

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This should allow good confidence in both the PPV and NPV with as few false test results as possible.

15. Keeping FPs to a minimum is a key concern, given their effect both on individual donors and on the blood supply. Even using an assay with sensitivity and specificity of 0.999, with a prevalence of 1 in 10,000, the PPV is only 9.08% (though the NPV is essentially 100%). To repeat the initial example, this translates into 250 true positive donations with 2,500 FP results.

16. The expected performance of existing serology assays used for primary screening by NBS is 0.95 sensitivity and specificity. At prevalence ranging around 1 in 100,000 donations or lower for the mandatory markers and Repeat Reactive rates of around 0.2%, the NBS can manage the losses of donations which arise from FP results. It is critical that NBS does not initiate screening for vCJD with assays that do not match the performance demanded of the serology tests. Using assays of lower specificity would lead to greater numbers of FP results and so to discarding donations at the numbers shown in Table 1.

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Extract from WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006).

Minor P *et al*. (2004). Standards for the assay of CJD disease specimens. *J.Gen.Vir* 85, 1777-1784.

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Extract from WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006).

Approach to validation of tests: strategies for development of reference materials.

P Minor

Following decisions reached at the WHO Consultation on Diagnostic Procedures for TSE in 1999 (Asher, Padilla et al. 1999), and under the direction of the WHO Working Group on TSE Reference Materials, the WHO sponsored the preparation of candidate reference materials consisting of 10% w/v homogenates of brain tissues from two sporadic cases of CJD, one case of vCJD, and one normal control brain—all from persons homozygous for methionine at codon 129 of the PRNP gene. Results of an international unblinded collaborative study using the homogenates and conducted by six research groups, comparing the analytical sensitivities of immunoblotting assays for PrPTSE, supported the establishment of these materials as WHO Reference Reagents (WHO 2003; Minor, Newham et al. 2004). Subsequently, assays of infectivity were performed using the same WHO sCJD and vCJD brain Reference Reagents in a line of mice transgenic for a humanized PrP-encoding gene yielded endpoint titres (Cervenakova, unpublished) similar to those reported for PrPTSE using a modified conformation-dependent immunoassay (Bellon, Seyfert- Brandt et al. 2003; Polymenidou, Stoeck et al. 2005). The WHO Reference Reagents are available from the National Institute for Biological Standards and Control (NIBSC), UK and are intended for use in small quantities as calibrants for laboratory working stocks, not as seed materials or for use in spiking studies.

More recently, the NIBSC also prepared samples of two vCJD spleens and one normal spleen, a brain suspension from a second vCJD case, and a brain from a case of sCJD in a patient heterozygous for PRNP-129 methionine and valine. Two dilution series from suspensions of vCJD brain and vCJD spleen have been spiked into human plasma; four laboratories have examined these materials. Further studies are under way.

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Extract of SEAC 91 minutes on methods to evaluate the efficacy of prion reduction filters.

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SPONGIFORM ENCEPHALOPATHY ADVISORY COMMITTEE
Minutes of the open session of the 91st meeting held on 24th February 2006

ITEM 6 – METHODS TO EVALUATE THE EFFICACY OF PRION REDUCTION FILTERS (SEAC 91/3)

1. The Chair explained that the UK Blood Service (UKBS) had previously asked the committee's advice about the implementation of prion reduction filters as a blood safety measure. SEAC had recommended that UKBS commission an independent validation of the filters and produce an assessment of the potential effectiveness of the filters to reduce transmission risks. The UKBS Prion Reduction Group has asked for the committee's input into the methodologies used to validate the filters.

2. Dr Marc Turner (UKBS) explained that two companies were developing prion reduction filters designed to remove prions from the red blood cell concentrate (RBC) of leucodepleted blood, Pall Medical Corporation (Pall) and Pathogen Removal and Diagnostic Technology Incorporated (PRDT). Pall has a CE mark[17] for its filter and the PRDT filter is expected to have a CE mark in the near future. Following SEAC's recommendation, UKBS had initiated an independent evaluation of the filters with two strands. One strand would evaluate the quality and safety of the filtered blood product. This would involve clinical studies and take about 18 months to complete. The second strand would evaluate the efficacy of the filters in removing prion infectivity. Due to the lack of sufficient blood samples of defined vCJD infectivity, it would not be possible to validate the efficacy of the filters directly. Three studies to evaluate the filters were proposed:

   (i) Measurement of the efficacy of the filters in reducing the infectivity in human leucodepleted RBC spiked with hamster scrapie brain, either as crude brain homogenate, microsomal fractions or sonicated microsomal fractions by biochemical assays and hamster bioassay. These experiments would allow the filters to be assessed using the same methodology as the companies. In addition, this approach would enable any substantial reduction in infectivity to be

[17] A declaration by the manufacturer that a product meets all the necessary requirements of the relevant legislation.

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measured. This is important since risk assessments suggest that a 3-4 $\log_{10}$ reduction in infectivity would be required to significantly reduce transmission risks.

(ii) Measurement of the efficacy of the filters in reducing the infectivity in human leucodepleted RBC spiked with splenic homogenate of mouse-adapted BSE. This would assess the efficacy of the filters in reducing the infectivity of a TSE strain more closely related to the vCJD strain in a different species. Additionally, different forms of homogenate would be used. These experiments should provide an indication of the utility of the filters to reduce infectivity of more than one prion strain.

(iii) Assessment of the filters in reducing endogenous infectivity in blood, recognising that brain and spleen homogenates are unlikely to represent the true physico-chemical nature of vCJD infectivity in blood.

3. Dr Turner explained that proposals had been invited to undertake studies (i) and (ii). These studies might start in summer 2006 with results available from the end of 2007. UKBS requested SEAC's advice at this stage on the suitability of studies (i) and (ii), guidance on the necessity and the model(s) that should be used in study (iii) and any additional work that would be useful to undertake.

4. Members welcomed the UKBS approach, in particular:

- the number of multiple filtrations that would be undertaken in the spiking experiments noting that in the published study by Pall only one filtration had been conducted,
- the selection of the 2 strains of TSE agent, noting that one was the widely used hamster scrapie strain on which there is an abundant literature. The second would be a BSE strain, which was of more relevance to the human situation.
- the high infectivity titres to be used allowing the filters to be tested over a wide dynamic range.
- the use of three different spiking materials allowing the filters to be tested on different types of preparations.

5. Members considered it important that study (i) replicated the companies' studies as closely as possible. Dr Turner explained that Pall and PRDT would be invited to prepare detailed dossiers of their work and to present their work to UKBS and the organisation selected to carry out study (i).

6. A member asked why the filters would only be tested using leucodepleted RBC since it was possible that the filter might not operate equivalently on

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leucodepleted and non-leucodepleted blood. Dr Turner explained that the filters were to be used in addition to leucodepeletion. Members recommended that the filters be evaluated on both leucodepleted and non-leucodepleted blood, since if they worked well on non-leucodepleted blood it may be possible to remove the leucodepletion step.

7. Members noted that the specification did not demand that experiments be conducted to good laboratory practice (GLP). Dr Turner explained that although it was not possible to conduct the work at full GLP because of the nature of some of the materials and protocols, it would be carried out to the highest possible standards and in the sprit of GLP. Members suggested that UKBS ask for the studies to be conducted to GLP with specified exemptions.

8. Members considered it necessary to conduct studies using endogenous infectivity in blood as it was crucial to use a model that reflected as closely as possible the human situation. It was noted that such experiments are difficult to conduct because of the difficulty in testing low levels of infectivity over a small dynamic range. Cost and experimental practicalities would need to be considered when selecting the most appropriate model but in scientific terms it was noted that:

   - rodent models allow bioassays to be conducted on the filtered material in the absence of a species barrier. The relatively short incubation period of TSEs in rodents would be advantageous. In addition, rodents have been used extensively in TSE studies so their characteristics are relatively well understood. However, the small volumes of blood that can be collected may be problematic. For example, given the known infectivity in hamster blood of about 10 ID_{50}/ml, the blood from about 250 clinically infected hamsters would need to be collected for filtration and inoculation to measure a 4 $\log_{10}$ reduction in infectivity. In mice with mouse adapted vCJD, the infectivity in blood is about 20 ID_{50}/ml. Humanised mice may be a better rodent model more closely reflecting the human situation, but the infectivity level in blood of such mice is unknown and the incubation period in these animals varies widely.
   - Non-human primates are the model that most closely reflects the human situation and large amounts of blood could be obtained from each animal. However, experiments would take a long time to complete. In addition, the infectivity titre in the blood of non-human primates was unknown. Experiments were being undertaken to investigate the vCJD infectivity titre in the blood of non-human primates but results would not be available for several years. Non-human primates could not readily be used as bioassays. Although humanised mice could be used for this purpose their utility as

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bioassays for primate blood would need to be assessed. Use of non-human primates reflects the human situation most closely but raises cost as well as having ethical implications.

- Sheep had also been used as a useful model to assess transmission via blood. Ovinised mice could be used as bioassays for sheep blood and their utility for this purpose is currently being assessed.
- Development of cell based assays to detect TSE infectivity should be encouraged.

9. Members asked whether it would be possible to use blood from patients with vCJD as a final test of the filters. Humanised mice could be used as bioassays to assess the reduction in infectivity. Such experiments could provide information on the infectivity in human blood. Dr Turner explained that small amounts of human blood from patients with vCJD were available but to test a filter a whole unit of blood (450 ml) would be needed. Dr Stephenson noted that there were ethical concerns around the collection of blood from patients with vCJD, and the use of non-human primates, but recommendations from SEAC would strengthen applications to do such work. Members suggested that blood from individuals considered 'at risk of vCJD' should be collected with ethical approval and patient consent.

10. A member suggested that, given the difficulties in detecting infectivity in filtered blood, it might be easier to analyse the material retained on the filter. In the future, it might be possible to test this material to assess infection in individuals and ascertain the prevalence of infection from studies of large number of individuals. Infectivity studies could be conducted on the material collected by the filters.

11. A member suggested that provided the safety and quality issues had been addressed, and the results of studies (i) and (ii) demonstrated the filters to be effective, it might be possible to start using the filters before the results of study (iii) were known. Dr Turner agreed and noted that the Committee on Microbiological Safety of Bone, Tissue and Organs would need to consider this issue. Members agreed that ascertaining the prevalence of vCJD infectivity in the populations was a critical factor in this consideration.

12. Dr Peter Bennett (DH) noted that in evaluating the effectiveness of filters to reduce transmission risks it was important to know the starting infectivity in specific blood components. There is presently a wide range of scenarios of infectivity. For example, it has been thought that infectivity may reside mainly in leucocytes. More recently it has been suggested that infectivity may reside almost solely in plasma. These different scenarios have a direct impact on the

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assessment of the effectiveness of the filters. It is envisaged that infectivity in specific blood components would be discussed at a future SEAC meeting.

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