

# WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies



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# EXECUTIVE SUMMARY

A variant form of Creutzfeldt-Jakob Disease (vCJD), a fatal brain disease of humans, first appeared in the mid-1990s, as a result of the bovine spongiform encephalopathy (BSE or “mad-cow” disease) epidemic in the United Kingdom. Since first reported in 1996, up to June 2006, 161 cases of vCJD have occurred in the United Kingdom, 17 in France, four in Ireland, two cases in USA and the Netherlands and single cases in Canada, Italy, Japan, Portugal, Saudi Arabia and Spain. Cases of BSE and vCJD have been decreasing in the United Kingdom in recent years, but both diseases have appeared in other countries.

Until recently, all vCJD cases were attributed to consumption of beef products contaminated with the infectious agent of BSE. Since December 2003, three individuals have been identified with vCJD infections probably acquired from blood transfusions – two with typical vCJD symptoms and the other with pre-clinical vCJD involving spleen and lymph nodes but not brain. The fact that the three vCJD infections followed transfusions from clinically healthy persons who became ill more than a year after donating blood implies that other blood donors who might currently be incubating the disease would also be potential sources of infection for recipients. The possible extent of future blood-borne spread of vCJD infections is still unknown. The identification of these cases has intensified the concern about possible unmapped ways in which the disease might spread. Except for the three transfusion-transmitted infections, no cases of vCJD have been linked to any medicinal product to date, and guidelines have been developed by the World Health Organization (WHO) and other authorities to minimize the risk.

A Consultation held at the WHO in September 2005 brought together experts and regulators from around the world to revise existing WHO Guidelines on Transmissible Spongiform Encephalopathies (TSEs) in relation to Biological and Pharmaceutical Products (2003) which recommended ways to prevent potential transmission of vCJD through human blood and blood products, as well as through medicinal products prepared with bovine derived materials. The primary aim of the Consultation was to provide evidence-based information to national regulatory authorities of WHO Member States – especially to those where BSE has not yet been reported and where surveillance systems for BSE and vCJD may not be in place. The information is intended to assist them in conducting risk assessments and selecting measures to reduce the risk of transmitting vCJD by human blood and blood products and other medicinal products of biological origin, collectively called biologicals.

The WHO Guidelines of 2003 encouraged authorities to consider introducing precautionary measures to minimize the then-theoretical risk of transmitting vCJD by blood and blood products while not compromising an adequate supply. While acknowledging that transfusion-transmitted vCJD is no longer just a theoretical possibility, experts in the 2005 WHO Consultation again advised authorities to assess the vCJD risk in the context of their own national situations, weighing the potential benefits of adopting precautionary policies to reduce that risk against the impact that those policies might have on the supply of blood.

Earlier WHO Consultations repeatedly stressed that, when feasible, tissues or body fluids of ruminant origin should be avoided in the preparation of biological and pharmaceutical products. When bovine materials must be used, they should be obtained from sources assessed to have negligible risk from the infectious agent of BSE. Most bovine tissues, including bovine muscle, used to manufacture biologicals, if carefully selected by taking into account the geographical distribution of BSE and collected according to guidelines, have little risk of contamination with BSE agent. Recent findings of disease-associated proteins in muscles of sheep with scrapie (a disease similar to BSE but not known to infect humans) and the recognition of BSE itself in a goat, reinforce the need for manufacturers of biologicals to maintain the precautionary safety measures recommended in the previous WHO guidelines. Ruminant blood and blood derivatives, such as fetal calf serum

in cell cultures media and bovine serum albumin stabilizers, are also used to prepare biologicals. Bovine blood has not been identified as a source of infection, and properly collected fetal bovine serum has a negligible risk. However, the blood of sheep with experimental BSE or natural scrapie can be infectious and, because scrapie and BSE agents behave similarly in sheep and goats, the blood of small ruminants should either be avoided in preparing biologicals or selected very carefully from sources known to be free of TSEs.

There is a continuing need to ensure that all national regulatory authorities with limited resources have ready access to reliable and up-to-date information when assessing TSE risks and evaluating product safety. That information includes guidance to help minimize or eliminate the risk for transmitting BSE and vCJD to humans via biologicals and other medicinal products.

# INTRODUCTION

The WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products, published in 2003, (<http://www.who.int/bloodproducts/TSE/en>) defined major categories of TSE infectivity, both in human and animal tissues, and have formed the basis of worldwide regulations for biological and pharmaceutical products. Preventive measures to minimize the risks of transmission to humans from both animal and human derived materials were also included in those Guidelines. New scientific information regarding the distribution of infectivity in various tissues from different species affected with a transmissible spongiform encephalopathy (TSE or prion disease) has emerged during the last three years. Some of these findings have challenged the current understanding of the tissue distribution of the pathological misfolded prion protein (PrP<sup>TSE</sup>)<sup>1</sup> and tissue infectivity. An example of these is the finding of PrP<sup>TSE</sup> in sheep muscle. This is the first evidence of PrP<sup>TSE</sup> in muscle from an animal species that enters the human food chain.

Also of concern has been the identification of three cases of probable transfusion-transmitted vCJD<sup>2</sup>, suggesting that secondary transmission of vCJD from human to human has occurred. The demonstration of vCJD transmission via blood creates a special concern for those countries that have no human TSE surveillance system in place. Blood donors subsequently diagnosed as suffering from vCJD have been identified in the UK, France, Ireland, Saudi Arabia and Spain. It is clear that the blood of donors incubating vCJD might contribute to an unrecognized spread of the disease, especially in countries where surveillance and reporting systems are not established. This new concern about human-to-human transmission should not distract from efforts to estimate and reduce the well established risk of food-borne BSE agent leading to vCJD cases. Each country should evaluate the risk of vCJD from both sources.

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.

In order to review and summarize the latest data on the epidemiology of vCJD, and on the detection of the infectious agents in blood, as well as the distribution of infectivity (and the abnormal prion protein associated with infectivity) in other tissues or body fluids of relevant species with TSEs, a Consultation of international experts was convened at WHO in Geneva on 14-16 September 2005. The analysis of the information presented provided scientific evidence to support the updating of the WHO Recommendations published in 2003. Experts at the Consultation were also asked to consider whether additional measures should be recommended to maintain the safety of blood and blood products, vaccines and other medicinal products with respect to TSEs.

Policies developed to reduce the risks resulting from the hazard of vCJD and its presumptive human-to-human transmission are based on three main factors: (a) an unknown number of individuals have been infected with the BSE agent; (b) the pathological misfolded prion protein found in TSEs – here designated as PrP<sup>TSE</sup> – and infectivity, detected by bioassay, are present in some peripheral tissues of patients who died of vCJD; (c) blood of rodents, sheep and possibly non-human primates infected with TSE agents has transmitted disease experimentally and, three persons in the UK have very probably been infected with the vCJD agent from the blood of asymptomatic donors who later died of vCJD. There is increasing concern that, without appropriate controls, blood products and other pharmaceuticals manufactured using bovine-derived or human-derived materials might spread the agent of vCJD worldwide, even to countries where BSE has not been reported. There is, therefore, a need to ensure that regulatory authorities worldwide have reliable information to assess the risk of human exposure to the BSE/vCJD agent, so that steps can be taken to prevent the transmission of TSE to humans via biologicals and other pharmaceutical products.

<sup>1</sup>Because of increasing complexity in terminology for various forms of the prion protein (PrP), the Consultation used the term PrP<sup>TSE</sup> for all abnormal misfolded PrP associated with TSEs.

<sup>2</sup>The third case was reported in February 2006 (<http://www.eurosurveillance.org/ew>)

# REVIEW OF SCIENTIFIC DEVELOPMENTS

Changes in the distribution and size of the BSE epidemic in Europe and elsewhere continue to be observed. The distribution of infectivity in tissues and body fluids in sporadic CJD (sCJD), vCJD, BSE and scrapie has been better established and methods for detecting the PrP<sup>TSE</sup> in different tissues have improved. Summaries of the information reported by the experts in the Consultation together with the relevant scientific references are provided in Annex 2.

## Epidemiology, clinical features and diagnostic criteria of Creutzfeldt-Jakob disease (CJD)

CJD is a rare and fatal human neurodegenerative condition. Like other TSEs, CJD is experimentally transmissible to animals, and a characteristic spongiform change is seen on microscopic examination of the brain. Epidemiological studies indicate a worldwide occurrence of sporadic disease of approximately 1-2 cases per million people per year. Globally, over 80% of cases of CJD occur as a sporadic disease (sCJD). Familial, iatrogenic, and variant forms of CJD show much lower and variable incidence in different countries. Most cases of vCJD have been found in the UK.

The origin of sCJD remains unknown despite extensive study. In particular, there is no evidence of a causal link with scrapie, a naturally occurring TSE of sheep and goats, or with BSE. Most sCJD cases occur in persons between the ages of 60 and 80 years with an average age at death of about 67 years. The typical patient with sCJD develops a rapidly progressive dementia associated with multifocal neurological signs, ataxia, and myoclonus. Although, in the correct clinical context, a characteristic EEG recording and/or the detection of 14-3-3 protein in the cerebrospinal fluid are considered diagnostic, confirmation of the diagnosis of CJD still relies on neuropathological examination. The clinical and histopathological features of sCJD are variable and are influenced by a naturally occurring polymorphism at codon 129 of the gene encoding the prion protein (PRNP gene). A novel test based upon the detection of PrP<sup>TSE</sup> in the nasal olfactory mucosa may improve the diagnosis of sCJD.

Familial CJD, also experimentally transmissible, is expressed as an autosomal dominant trait associated with one of several abnormalities of the PRNP gene in different affected kindreds. Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI), similarly inherited neurodegenerative disorders linked to mutations in the PRNP gene, have also been transmitted to animals.

There have now been at least 362 recognized cases<sup>1</sup> of iatrogenic CJD following use of the following products: contaminated human-pituitary-derived growth hormone (180 cases) and gonadotropin (four cases), human dura mater allografts (168 cases), corneal transplants (at least three probable or possible cases), neurosurgical instruments (four or possibly five cases) and a stereotactic cortical-probe EEG electrode (two cases). Except for the three transfusion-transmitted vCJD infections described above, no new class of products causing iatrogenic CJD has been identified during the last nine years, although the number of cases resulting from past exposures to known products continues to increase.

Since first reported in 1996, up to June 2006, there have been 161 cases of vCJD in the United Kingdom, 17 in France, four in Ireland, two in the USA and in the Netherlands and single cases in Canada, Italy, Japan, Portugal, Saudi Arabia and Spain. Cases of BSE and vCJD have been decreasing in the United Kingdom in recent years, but both diseases have appeared in other countries and the notification rate for new cases of vCJD has increased in France during the past two years. The US, Canadian and two of the Irish patients had spent several years in the UK

<sup>1</sup>The numbers of all iatrogenic cases were kindly provided by Dr Paul Brown (October 2005)

between 1980 and 1996, and were probably exposed to the BSE agent there, while the Italian, the Dutch cases and 16 of the 17 French cases had no history of significant travel outside their home countries. The median age at onset of vCJD is 26 years (range 12-74 years) with a median duration of illness of 14 months (range six to 40 months). Although the definitive diagnosis of vCJD requires neuropathological examination, clinical and laboratory criteria have been established to diagnose probable vCJD in living patients. All clinically manifest vCJD cases (191 worldwide) tested were homozygous for the allele encoding methionine at codon 129 of the PRNP gene. A distinctive feature of vCJD—in contrast to sCJD—is the frequent occurrence of PrP<sup>TSE</sup> in lymphoid tissues (tonsil, spleen, lymph node, and appendix). In three patients who underwent appendectomy before onset of vCJD, two had immunoreactive PrP<sup>TSE</sup> in lymphoid follicles of the appendix—one eight months and the other two years prior to death. One appendix was negative for PrP<sup>TSE</sup> nine years prior to death. An anonymous survey of surgically removed tonsils and appendices in the UK revealed three out of 12,674 cases which stained positively for PrP<sup>TSE</sup>. All three were appendices. Genetic studies on DNA extracted from two of the three positive appendices found that they were valine homozygous at codon 129 in the PRNP gene, unlike any of the clinical case of vCJD encountered so far.

The causal link between vCJD and BSE is based on epidemiological, biochemical and transmission studies. A Joint Technical Consultation on BSE 2001 convened by the WHO, the Food and Agriculture Organization (FAO) and the Office International des Epizooties (OIE, the World Organization for Animal Health) reached a scientific consensus that consumption of beef products contaminated with the BSE agent is the main avenue of exposure (<http://www.who.int/zoonoses/diseases/en>); this conclusion is still generally accepted. Bovines, bovine products and by-products potentially carrying the BSE agent have been traded worldwide, giving this risk a global dimension. Epidemiological analysis does not indicate that most medicinal products, including plasma-derived products, or occupational exposures have been sources of infection in vCJD cases identified to date. Three cases of vCJD infection presumptively transmitted by transfusion of red blood cell concentrates have been reported.

## **Bovine spongiform encephalopathy (BSE) and scrapie**

BSE was first identified in British cattle in November 1986. Current evidence suggests that the disease originated from the use of feed supplements containing meat-and-bone meal contaminated with a TSE agent (probably from scrapie-infected carcasses). In the UK, 184,370 confirmed cases of BSE had been reported by 31 December 2005 ([http://www.oie.int/eng/info/en\\_esbru.htm](http://www.oie.int/eng/info/en_esbru.htm)). Smaller outbreaks have been reported in native-born cattle in most other Western and Central European countries and in Canada, Israel, Japan, and the USA. Outside the UK, 5,428 confirmed cases had been reported to the OIE or the EC by 31 December 2005. Most recent cases were recognized either in cattle at increased risk for BSE (fallen stock and emergency slaughter cattle over 24 months old) or in clinically unremarkable slaughter cattle over 30 months old identified by statutory or other “rapid” testing (see Diagnosis) of brainstem tissue for PrP<sup>TSE</sup>. Such tests were introduced in Switzerland in 1999 and in the European Union in 2001. Active testing of all cattle was instituted in Japan after 2001 and in high-risk animals in Canada and the USA after 2003. Such targeted active surveillance in Europe has resulted in the better detection of infected animals during the pre-clinical and clinical stages of illness. In the UK, the incidence of BSE has continued to decline since 1992-1993, consequent to a statutory ruminant feed ban introduced in 1988 and reinforced in 1996. This decline is consistent with the hypothesis that BSE cases arose by infection from contaminated feed. Although epidemics of BSE in other European countries were recognized more recently than that in the UK, most are also in decline, and, so far, no single country except the UK and Ireland has reported more than 1500 cases.

In naturally affected cattle, BSE infectivity, detected by assay in mice, has been demonstrated only in the brain, spinal cord and retina. Assays of infectivity in cattle have also detected infectivity in a pool of nictitating membranes but not in pools of lymph nodes or spleen. Recently, infectivity was detected in some peripheral nerves and a solitary muscle, of a single case of BSE in a

German cow, assayed using highly sensitive transgenic mice over-expressing bovine PrP, suggesting a very low titre of infectivity.

In cattle experimentally exposed by the oral route, BSE infectivity has been detected by mouse assay in the distal ileum through much of the disease course from six months post exposure and in the central nervous system (CNS) and in sensory ganglia of the peripheral nervous system from late in the incubation period. Infectivity has also been detected in sternal bone marrow in cattle experimentally exposed to BSE agent by the oral route, but only at a single time point during clinical illness. Assays in cattle of selected tissues from this same initial sequential time point oral exposure study confirmed infectivity in distal ileum (from six through 18 months after exposure and during clinical disease) and in the CNS at the earliest time post-exposure detected by the mouse assay but not before. Infectivity has also been found in palatine tonsil, at a single time point in incubation, but this was detected only by assay in cattle and not by the mouse assay. A wide range of other tissues, including most lymphoreticular tissues, from cattle with BSE—both naturally acquired and experimentally induced—and from cattle in the incubation period after experimental exposure, contained no infectivity detectable by conventional mouse bioassays or ongoing parallel bioassays in cattle. Bioassays of bovine tissues injected into transgenic mice over-expressing bovine PrP, presumably reducing the PrP-associated species barrier, also support a conclusion that there is a limited distribution of BSE infectivity in bovine tissues.

BSE has been experimentally transmitted via the oral route to sheep and goats, and there is recent evidence that one goat has been naturally infected. Concern over the possibility of BSE in small ruminants led to increased efforts at active and passive surveillance of scrapie in the EU, based on the observation that experimental BSE in sheep and goats resembles scrapie. Recently, infectivity was found in blood of sheep with natural scrapie and in blood of sheep with experimental BSE during both the incubation period and clinical illness.

## Diagnosis

The accumulation of PrP<sup>TSE</sup> occurs only in TSEs, so its detection can serve as a surrogate for detection of infectivity in biological samples. After experimental inoculation of rodents with TSE agents, PrP<sup>TSE</sup>, like infectivity, is usually detectable in the CNS weeks before the appearance of overt disease, and its level increases during clinical illness. While the increase in PrP<sup>TSE</sup> generally parallels that of infectivity, the precise relationship between PrP<sup>TSE</sup> and infectivity is unclear. For example, under specific experimental conditions, the brains of some TSE-affected rodents may be infectious by bioassay while PrP<sup>TSE</sup> remains undetected. From the perspective of pre-clinical diagnosis, both the sensitivity of diagnostic methods and procedures to concentrate PrP<sup>TSE</sup> are crucial, because the amount of PrP<sup>TSE</sup> outside the CNS is likely to be small, particularly in circulating blood. PrP<sup>TSE</sup> can be concentrated by physicochemical precipitation, affinity precipitation techniques or affinity chromatography. Moreover, it has been reported that the amount of PrP<sup>TSE</sup> in dilute solutions can be increased considerably by the “protein misfolding cyclic amplification” (PMCA) technique, potentially allowing improved detection of extremely small amounts of infected material. In recent reports, the test developer described an improved PMCA that detected PrP<sup>TSE</sup> in the blood of most hamsters with scrapie and not in the blood of uninfected hamsters. In addition to blood, other readily accessible tissues might offer the possibility for diagnosis of clinical TSE. Tonsil biopsy has been used to diagnose vCJD in a minority of patients after the onset of clinical signs and symptoms. Also, a study that demonstrated PrP<sup>TSE</sup> and infectivity in the skeletal muscle of mice experimentally infected with laboratory strains of TSE has been at least partially confirmed by the detection of PrP<sup>TSE</sup> in skeletal muscles of small ruminants with TSEs and humans with both sporadic CJD and vCJD. These findings are under intense study by a number of laboratories.

Among immunological methods for PrP<sup>TSE</sup> detection, immunoblotting (Western blotting) is the most thoroughly characterized and widely used. Immunoblotting offers the advantage of recognizing different forms of PrP<sup>TSE</sup> through the analysis of the molecular mass, shift in electrophoretic mobility after digestion with proteinase K (PK), relative abundance of di-, mono- and non-glycosylated bands, and binding with a variety of epitope-specific monoclonal antibodies.

The size of PK-treated fully deglycosylated PrP and relative abundance of di-, mono- and non-glycosylated bands characterize the PrP type, a kind of PrP signature that varies among different forms of TSE. PrP<sup>TSE</sup> typing has been proposed for distinguishing various forms of TSE (e.g., scrapie from BSE and sCJD from vCJD) and for improving the classification of human TSEs.

ELISA and immunoblotting methods are commercially available in Europe, Canada and the USA as ready-to-use kits for postmortem animal diagnosis of TSEs; several of those tests (so-called “rapid” tests) have been validated in a study by the European Commission (EC) as screening tests for BSE in slaughtered cattle. EC-approved immunoassays have detected PrP<sup>TSE</sup> in the brains of BSE-infected cattle at least three months before onset of clinical illness. However, no immunological method has yet been validated to be sufficiently sensitive to detect PrP<sup>TSE</sup> in the blood of infected animals or humans, though promising initial results have been reported by several groups of investigators and were presented to the Consultation (see Annex 2). Some immunoassays have been claimed to detect PrP<sup>TSE</sup> in samples containing less than 1 LD<sub>50</sub> of BSE infectivity as measured by bioassay in transgenic mice expressing bovine PrP, though attempts to confirm the claims independently have not been successful.

## Risk of transmitting Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD) by human blood and blood products

Since the last WHO Consultation on this issue in 2003, new evidence relevant to risk assessments for the transmission of vCJD by human blood has accrued. Salient information is summarized here:

- (a) It has been known for more than 20 years that the blood of rodents experimentally infected with agents of several TSEs contains infectivity. Most recently, infectivity has been found in the blood of mice experimentally infected with the agent of vCJD.
- (b) There is convincing evidence that both scrapie and BSE can be transmitted from sheep to sheep by blood transfusions with either whole blood or buffy coat. Transfusions of blood from animals in the incubation period and clinical phase of illness have transmitted disease. Transfusion appears to be a relatively efficient mechanism for transmitting infection from sheep to sheep.
- (c) Epidemiological evidence indicates that vCJD infection has been transmitted to three recipients of blood transfusion. These three infected recipients demonstrate that blood contained infectivity during the latter part of the incubation period of vCJD, from 18 months to 3.5 years before the donors showed signs of neurological illness. The finding of three transfusion-transmitted vCJD cases among a relatively small number of persons transfused with blood components from vCJD donors, only about 18 of whom survived for more than five years, suggests that the transfusion of a human blood component has transmitted vCJD efficiently, an observation consistent with experimental animal studies. The first recipient developed vCJD 6.5 years after transfusion—considerably shorter than the probable minimum incubation periods of presumed food-borne cases of vCJD. The second recipient died without signs of neurological disease five years after transfusion but already had detectable PrP<sup>TSE</sup> in spleen and lymph nodes, though not in appendix, tonsil, or brain. The third patient, who developed clinically typical probable vCJD almost eight years after transfusion of red cells from a different donor, was still alive at the time of the Consultation in 2005 but has subsequently died.
- (d) To date, all cases of vCJD tested have been in persons homozygous for methionine at codon 129 of the prion-protein-encoding gene (PRNP gene). However, the transfusion recipient with pre-clinical or sub-clinical vCJD infection was heterozygous, having methionine and valine at PRNP codon 129, indicating that vCJD infection can occur in persons of this genotype, as can other forms of CJD. Furthermore, a study of anonymous tonsil and appendix specimens in the UK identified three instances in which appendix samples stained positively for PrP<sup>TSE</sup> using immunohistochemical techniques—although the staining pattern was described as being slightly different from that in lymphoid tissues of known vCJD cases. Genetic studies on DNA extracted from two of these three appendices found them to

- be valine homozygous at codon 129 in the PRNP gene; no DNA was extractable from the other specimen. Taken together, these findings suggest that, if exposed to a sufficient dose, most people are probably susceptible to infection with the BSE agent. In the UK, approximately 30% of Caucasian populations are homozygous for methionine at codon 129 of the PRNP gene, about 50% are heterozygous for methionine/valine, and the rest are homozygous for valine. The PRNP codon-129-valine allele is rarely found in East Asian populations.
- (e) The same tonsil-appendix survey results also suggest that a substantial number of individuals in the UK might be incubating vCJD—a mathematical analysis predicting that as many as 5,000 individuals in the total UK population (a rate of 237/million) might be infected. Some proportion of healthy individuals with sub-clinical or pre-clinical vCJD would presumably be blood donors. The possible prevalence of asymptomatic vCJD infections in other countries is not known.
  - (f) A probabilistic risk assessment model concluded that transmissions of infection by blood transfusion had a potential to increase the eventual size and duration of the current vCJD outbreak in the UK significantly. Deferral of transfusion recipients as blood donors was implemented in the UK in 2005; this step is anticipated to reduce substantially the risk of recycling vCJD infections. The same measure has been in place in France since 1998 and more recently in several other European countries such as Ireland, the Netherlands and Switzerland. In some other countries, like Canada, Australia, Italy and the US, blood donors previously transfused in the UK have been deferred.
  - (g) A risk assessment estimated that some derivatives prepared from pools of UK plasma were likely to have included one or more donations from persons incubating vCJD. That poses a small and uncertain risk of transmitting infection to some recipients of the products. The relative risk depends on the type of plasma product, the specific manufacturing process used and the year of production. The UK has no longer fractionated plasma of UK residents since 1999 and has imported all plasma for manufacture—most from the USA and some from Germany. Assessments suggested that vCJD risk from derivatives of plasma collected and manufactured in other countries was low or negligible.
  - (h) A few vCJD cases in the UK were in people previously transfused with blood components from donors not diagnosed with vCJD, and a risk assessment concluded that some of these recipients might conceivably have been infected through the transfusion. For two cases, the time between blood transfusion and onset of vCJD was so short that transfusion transmission seems highly improbable. Authorities have not concluded that any of the donors involved is sub-clinically infected with vCJD agent. Nevertheless, the implicated donors were informed that, as a precaution, they should no longer donate blood.

In conclusion, it is probable that vCJD has been transmitted through blood transfusion, with important implications for public health. Several vCJD cases have occurred outside the UK in persons who previously donated blood (in France, Ireland, Saudi Arabia and Spain). Authorities in those countries are aware of the potential risk. To date there is no evidence that vCJD has been transmitted by human plasma derivatives, in spite of intensive use of some products manufactured from plasma of UK donors during and after peak years of the UK BSE outbreak. But the incubation periods of TSEs can be very long, and possible transmission of vCJD by plasma derivatives, while not recognized to date, cannot be confidently excluded yet. Cumulative epidemiological evidence, including follow-up studies in Europe and the USA of more than a hundred long-term survivors transfused with blood components from donors who later developed other forms of CJD, suggests that the infectious agents associated with sporadic, familial and iatrogenic CJD have probably not been transmitted through blood or blood products, at least not with a frequency detectable by epidemiological surveys.

# RECOMMENDATIONS

## Tissue infectivity

The foundation of any attempt to construct a rational analysis of TSE risk for biological and other pharmaceutical products must begin with an evaluation of infectivity in the human or animal tissues from which these products are derived. Although straightforward in principle, the task is complicated by differences in the timing of first appearance and final tissue distribution of infectivity in different species and TSEs, by differences in the sensitivity of bioassay methods, and by incomplete data about infectivity levels in various tissues of interest. Tables IA, IB and IC in Annex 1 summarize current data about the distribution of infectivity and PrP<sup>TSE</sup> in humans with vCJD and other human forms of TSE, in cattle with BSE, and in sheep and goats with scrapie. In general, it can be said that, paradoxically, whereas both infectivity and PrP<sup>TSE</sup> in cattle with BSE have a more limited distribution in tissues than in any other animal or human form of TSE, PrP<sup>TSE</sup> and, to some extent, infectivity have a wider distribution in tissues of humans with vCJD than in other forms of CJD. In using the tables, it is important to note that two classes of material were intentionally excluded: (1) materials like bile of ruminants and humans that have never been studied, and (2) highly processed chemically pure reagents like tallow derivatives and bovine bone gelatin produced by the alkaline process that have been studied and found to pose a negligible risk if any for transmitting infection.

Several new methods attempting to detect PrP<sup>TSE</sup> using novel techniques (see Diagnosis and Annex 2), if successfully developed, might eventually offer sufficient sensitivity to demonstrate amounts of agent below the level of detection of currently validated tests. It has been speculated that such methods might find small amounts of agent in some tissues currently thought to be free of infectivity. It remains unknown whether tissues containing such very small amounts of infectious material would transmit infection to humans.

## Measures to minimize risks to humans from biological and pharmaceutical products in which ruminant materials are used during manufacture

Bovine materials are commonly used in the manufacture of many biological and pharmaceutical products. The use of ovine and caprine materials—except for the occasional use of sheep and goat antisera and the milk of transgenic goats—is relatively uncommon. On the basis of current scientific knowledge about the agents causing BSE and other animal TSEs, an ideal strategy would be to avoid altogether the use of ruminant (especially bovine) materials in the manufacture of any biological or pharmaceutical product, as well as the use of materials from other animal species in which TSEs naturally occur. In practice, this is often not feasible, in which case measures should be taken to minimize the risk of incorporating an infectious TSE agent. A risk assessment should be performed for the final product. The risk assessment should take into account three general factors: source of starting materials, manufacturing process and clinical use of the final product.

The sources of starting materials used as active substances, excipients or manufacturing reagents and their potential infectivity are most important. An assessment should include both the geographic source (country), history of the source animals (age, feeding, traceability), and details of the actual tissues used (risk of intrinsic infectivity in the tissue and risk of cross-contamination with higher-risk materials during slaughter or processing of tissue). It is generally acknowledged that source tissues processed using validated methods are likely to pose a lower risk than unprocessed or minimally processed source tissues. This is especially true when the processing conditions have a validated robust capacity to inactivate or remove TSE agents, such as those used in the manufacture of amino acids, tallow derivatives, and gelatin. For example, gelatin prepared from bovine bones—excluding skulls and vertebrae—subjected to a series of partially effective but additive processes such as hot-water washing under pressure, demineralization with acid, alkaline hydrolysis

and, especially, terminal ultra-high-temperature flash heat sterilization of the final product poses little risk.

An assessment of risk should consider the ability of a manufacturing process to inactivate infectivity or to remove infectivity by partition, as well as the possibility of cross contamination—both when starting materials are collected and processed and during manufacture of the medicinal products themselves. The production processes for preparing master and working viral and bacterial seeds as well as cell banks and other materials used in the manufacture of vaccines should not be neglected.

In considering the clinical use of the product in a risk assessment, both the route of administration and amounts of product likely to be used in a single course of therapy, in a year or even in a lifetime should be taken into account. A thorough TSE risk assessment contributes to the overall risk-benefit analyses for biological and other pharmaceutical products.

### **Source of starting materials**

Careful selection of the source of ruminant starting materials used to manufacture active substances, excipients and in-process reagents is an important consideration in the TSE risk assessment. The most satisfactory source of materials is from countries where the risk of BSE in cattle is low and adequately controlled. Countries are encouraged to assess geographic BSE risk, and the OIE offers guidance in that process through the latest edition of the OIE Terrestrial Animal Health Code in its chapter on BSE ([http://www.oie.int/eng/normes/mcode/A\\_summary.htm](http://www.oie.int/eng/normes/mcode/A_summary.htm)). OIE currently suggests that countries assign themselves to one of three categories of BSE risk: negligible, controlled or undetermined. National regulatory authorities may use their own more refined estimates of BSE risk for purposes of import controls. For the EU, the Geographical BSE Risk categorization—a standard qualitative approach to assess the risk of BSE for cattle in countries submitting requests to trade—has been expanded and updated continuously, originally by the Scientific Steering Committee of the EC (Health and Consumer Protection Directorate General) and subsequently by the European Food Safety Authority (EFSA, at [http://www.efsa.eu.int/science/tse\\_assessments/catindex\\_en.html](http://www.efsa.eu.int/science/tse_assessments/catindex_en.html)). The Geographical BSE Risk categorization recognizes the important fact that if the prevalence of BSE in a country varies over time so too will the result of its risk assessment.

The use of ruminant source materials from countries with an undetermined risk of BSE is usually not acceptable. However, even in those countries, the collection of source materials for the manufacture of specific products from well-monitored herds might sometimes be allowed, particularly if they are from tissues appearing in Table IC and they are collected from healthy cattle in a safe manner. This should be done only if evidence is provided that the herds have had no cases of BSE, and have implemented an active BSE surveillance program. In addition, these ruminants should have never been fed meat-and-bone meal nor any other mammalian-derived proteins prohibited by national authorities (including some processed animal proteins) and have a fully documented breeding history, including introduction of new genetic material only following OIE guidelines for international trade in bovine semen and embryos.

The age of animals from which tissues or fluids are collected as starting materials should also be taken into account. Studies on infectivity of tissues collected during the incubation period of experimental BSE in cattle showed that tissues of younger animals generally contained less infectivity than did those of older animals. However, some bovine tissues, like ileum and tonsil, contained infectivity at an early stage following oral exposure to the BSE agent. Such tissues may be considered safe only where the risk of BSE in cattle is negligible or where epidemics of BSE have been in continuous decline and as long as rigorous high-quality surveillance and BSE control procedures remain implemented.

### **Tissue removal and processing**

Potential TSE risks might be influenced by circumstances under which tissues are removed. For example, both penetrative and some non-penetrative techniques for stunning cattle before terminal exsanguination can embolize brain tissue into the general circulation and increase the risk that

tissues containing little or no intrinsic infectivity (e.g., lung) might become contaminated with high-risk tissue. Stunning devices that inject air into the cranial cavity and the process known as “pithing” are especially dangerous in this regard. Methods to prevent cross-contamination of the carcass with brain from skull wounds and from neural tissue exposed during decapitation should be in place. Sawing of the vertebral column introduces another possibility for contaminating intrinsically low-risk tissue with spinal cord. Collecting selected tissues before the carcass is split greatly reduces that risk. Body fluids should be collected with minimal damage to tissue, and cellular components should be removed. Blood of young animals with known provenance is less likely to pose the risk of cross-contamination. Fetal blood should be collected without contamination from other maternal or fetal tissues, including placenta, and whenever possible, by taking blood by cardiac puncture with single-use instruments using closed collection systems for all materials. When potential cross-contamination of a source tissue with a tissue of higher risk cannot be reasonably excluded, a higher risk of infection must be assumed for purposes of risk evaluation. In short, particular care should be taken to avoid any kind of contact between collected materials and higher-risk materials. Some national authorities have defined certain bovine materials of greatest concern as “specified-risk” materials and required removal of those materials from carcasses.

Facilities that provide starting materials for medicinal products should have in place an appropriate quality system to document the process used and provide a record for each batch of starting material collected. They should either have or work towards official accreditation of the quality system. For example, the EU grants Certificates of Suitability for products complying with the EU Note for Guidance on TSE Risks, which stresses the need for a quality assurance system. Procedures should also be in place to reduce the risks of adulteration of batches.

The sources and types of starting materials, while important, are not necessarily the only determinants of risk of potential TSE transmission. Some manufacturing processes—for example those used to produce bovine serum albumin and tallow derivatives—have a substantial demonstrated ability to eliminate infectivity that might be present in the starting material. Processes that inactivate infectivity or remove infectivity from starting materials augment the safety provided by appropriate sourcing. Manufacturers should consider including such procedures in their manufacturing processes when possible. Claims that a production process contributes significantly to the safety of a product should be validated.

## Production systems

### *Vaccines*

Production systems also affect the final TSE risk assessment for vaccines. Many vaccines are prepared from organisms that cannot be treated with harsh methods of extraction or purification without reducing or destroying their antigenicity. Additional difficulties are inherent in the cell bank and seed lot systems employed in vaccines production. Concerns with respect to TSE may arise from the animals used for in vivo production or as a source of cells for production in vitro, from components of medium used in production, or from the cell banks or bacterial or viral seeds used to initiate production. Where vaccine strains are still grown in animals (such as vaccinia virus for smallpox vaccine), careful selection of source materials and, in some cases, postmortem testing of each production animal can greatly reduce the TSE risk. Some vaccines are produced in primary cell cultures, usually derived from species of animals not known to have TSEs. Such cultures are very unlikely to be infected or contaminated with TSE agents. Nevertheless, cells should be selected carefully, avoiding those known to replicate TSE agents. However, culture medium used to grow bacterial, yeast, mammalian or other cells in vitro may contain components of animal origin. A TSE risk assessment should be carried out for such production systems.

The most complex TSE issues are raised by banked eukaryotic or bacterial cells and viral vaccine seeds. The Consultation strongly emphasized that, by virtue of the level of characterization possible, the overall risk-benefit assessment overwhelmingly favors the use of banked cells and the seed lot system for vaccine production. However, TSE risk assessments of banked cells and viral or bacterial vaccine seed stocks should take into account the possible carryover of any potentially infectious material from the seed into the final product as a contaminant. There is also a theoretical

possibility that production cells might be infected with a TSE agent, although none of the very few cell lines known to support replication of a TSE agent has been used to produce any vaccine.

When evaluating the possibility of potentially infectious material in a seed, not all relevant information may be available, since some seeds were derived long ago and often have a lifetime of decades. Tracing their origin and full production history may be impossible. Where information is incomplete, it is recommended that, when feasible, working seeds be replaced as a precautionary measure, taking into account the need to maintain adequate supplies of vaccines with public health benefits during the replacement. For existing products, master seed materials and original experimental preparations from which master seeds are derived (“pre-master” seeds) may not need replacement, since the biological phenotype of the vaccine depends on these materials, and they may be difficult or impossible to recreate. However, the goal of eventually replacing seeds with those of impeccable provenance for all reagents should not be abandoned. The origin of newly developed products should be documented as completely as possible, recognizing that this may also be difficult. For new products made using old seed lots, any existing risk assessment for the seed and history of prior use of the seed should be taken into account.

When deriving new vaccines with new cell banks or viral or bacterial seeds, developers should take into account all guidance on TSE risk in force at the time that laboratory work begins. However, since development of new vaccines often takes years, complete information on TSE risk for older seed materials may not meet requirements in force later, when a candidate new product must be assessed for licensure. The principles on seed materials outlined in the paragraph above should apply to such cases.

### ***Recombinant DNA products***

Medicines produced by recombinant DNA technology use a cell banking system similar to that used for many vaccines. Similar considerations with respect to production media, carryover of contaminants and the theoretical possibility of infection of the cells therefore apply. Risk assessments should take the same approaches used for vaccines.

### ***Other medicinal products***

A number of bovine-derived materials are commonly used to manufacture both biological and pharmaceutical products. These include milk and milk derivatives (like casein), meat extracts, bovine serum including fetal bovine serum, bovine bone gelatin, bile derivatives (deoxycholate, choline) and beef tallow derivatives (triglycerides, glycerol, sorbitol esters, polysorbates, other). Beef tallow prepared using specified-risk materials should be avoided. Materials originating from non-bovine ruminants are less commonly used, although substantial amounts of mixed-species tallow may be produced from some rendering plants. Infectivity of experimental BSE is more widely distributed in tissues of small ruminants (see Annex 1) than in cattle, posing a special concern.

Milk and certain milk derivatives, such as lactose, are generally considered non-infectious, regardless of geographic origin, provided that the milk is from healthy cows fit for human consumption and no other potentially infectious ruminant-derived materials were used in the manufacturing process. Rennet, derived from the abomasum of cattle—which has shown no detectable BSE infectivity (Annex 1, Table IB)—sometimes used during the manufacture of lactose is generally considered to pose no significant risk, especially when derived from calves.

Extracts prepared from tissues like bovine muscle, in which infectivity was recently detected during the clinical phase of BSE in one cow (Annex 1, Table IB) are unlikely to present more than a negligible risk of TSE contamination, provided that the manufacturer has scrupulously complied with procedures designed to avoid cross contamination with specified-risk materials during preparation of the source material. If assurances of compliance are not available, then it is recommended to source meat extracts from animals in countries where risk of BSE is remote. Recently, using tests of increased sensitivity, infectivity and PrP<sup>TSE</sup> were detected in peripheral nerves of cattle and, PrP<sup>TSE</sup> in enteric plexus. An additional safeguard for bovine muscle used to prepare vaccines or other biologics might be to ensure removal of all visible nervous and lymphatic tissue from muscle before collection and to avoid using meat from the tongue or the head. A simpler approach would be to source meat for nutrient broths from young animals in countries where the risk of BSE is negligible.

As noted above, TSE infectivity has been detected in transfused blood from sheep with natural scrapie and sheep experimentally infected with BSE. Transfusion experiments have not been conducted in cattle. Effects of blood clot formation on TSE infectivity in serum have not been established. Studies using small amounts of blood components or spleens of cattle with BSE assayed in mice and cattle injected by the most effective routes failed to detect infectivity. A conservative regulatory approach would assume that bovine serum might potentially contain TSE infectivity—presumably in small amounts. Blood for the preparation of donor calf serum is most often collected from well controlled living animals, reducing the risk of cross-contamination of blood with higher-risk materials attendant to the stunning and slaughtering process. Thus the sourcing of bovine serum (country/herd/animal) combined with appropriate precautions to avoid cross-contamination during collection is important in the risk analysis. An additional safeguard might be to collect blood from impeccable sources and to store separated components for a period of time exceeding the mean incubation period of cattle—at least five years. Such a strategy would increase confidence in the safety of the material if no BSE had been detected in the country.

Gelatin may be extracted from the skin and/or bones of cattle and pigs. Skins of either species and bones of pigs are likely to have a negligible TSE risk, provided that contact with bovine specified-risk materials is avoided. Gelatin from bovine bones originating anywhere except a country with negligible BSE risk should be produced by alkaline hydrolysis—including pressure washing with hot water, acid demineralization, and filtration, augmented, whenever possible, by an adequate flash ultra-high-temperature heat sterilization process—rather than by acid treatment alone. Bovine bones for gelatin should carefully exclude skulls and vertebral columns if obtained from countries other than those with negligible BSE risk. Compliance with these precautions provides assurance that gelatin used in the manufacture of medicinal products is unlikely to be contaminated. Amino acids derived from gelatin are further highly processed, so their risk may be even lower.

Materials derived from ruminant tallow (see above) and amino acids of ruminant origin, even if higher-risk tissues were not completely eliminated, are considered highly unlikely to remain contaminated by the time the final reagents have been produced, so long as the reagents were prepared by processes of extraction and purification at high temperature and pressure, and good manufacturing practices (GMP) were rigorously controlled. Safety is further assured when specified-risk materials are excluded from starting materials, when raw materials are pressure cooked according to the OIE Code (particle size  $\leq 50$  mm, temperature  $> 133^{\circ}\text{C}$ , pressure  $\geq 3$  bar, exposure time  $\geq 20$  min) and when proteins have been removed from tallow to meet OIE specifications. The OIE Code chapter on BSE provides specific guidance on requirements for safe trading in commodities used to manufacture biologicals and other medicinal products.

## Measures to minimize risks to humans from human-derived materials

### The risk of transmitting vCJD by blood and blood products

Although the UK remains the country at greatest risk from past exposure to the BSE agent, vCJD has also been identified in an increasing number of other countries in recent years. In most of those countries only single cases have been identified. But the occurrence of even one vCJD case means that other people may be at risk of primary food-borne infection, either through consumption of imported BSE-contaminated beef products or by consumption of infected beef products during travel to the UK or to other increased-risk countries before effective BSE control measures were in place. Furthermore, some countries without recognized cases of vCJD might have unrecognized BSE in cattle or have unknowingly imported products contaminated with the BSE agent. Therefore, it is likely that cases of vCJD will continue to occur, both in Europe and elsewhere. It would be prudent for national authorities to prepare, in advance, plans to reduce the risk of secondary transmission by blood components and plasma-derived products, even if BSE and vCJD have not been recognized in the country.

## Risk assessment

Blood, blood components and plasma-derived products are essential in medical treatment and can be life-saving. An effective health care system should strive to ensure a consistent and adequate supply of these products. Some measures to reduce the risk of vCJD, for example by deferral of certain donors, might compromise supplies of blood and plasma. It is essential that any measures introduced in response to vCJD be proportionate to the risks, which vary from country to country, while maintaining adequate supplies of blood-derived products.

A risk assessment pertaining to a particular country should be undertaken in order to recommend appropriate risk-mitigating measures to employ in particular circumstances. Several countries, including Australia, Canada, France, Germany, UK, and the USA, have undertaken risk assessments using various modeling techniques and assumptions. Such risk assessments provide a basis for examining the adequacy of different measures to minimize the risks to humans from human-derived materials and provide a framework for developing regulatory and public health actions. In some countries, risk assessments are carried out by expert groups independent from, but at the request of, the national regulatory agencies that recommend or select risk management actions. However, it is accepted that assessments cannot predict risk precisely, because of great uncertainties in the assumptions used. Risk assessments, while useful tools for developing risk-mitigating strategies, are not the only tool. New information on vCJD prevalence and accumulation of actual data on transmission by blood will inevitably allow refinement of risk evaluation. As new technologies, such as better testing for PrP<sup>TSE</sup> and practical procedures for reducing infectivity, are developed and validated, risk assessments can be further refined and risk-mitigating measures revised. Risk management should consider actions mitigating the consequences of both historical risk and current risk, which may need different approaches. Risk communication is another important component of policy but an aspect fraught with special difficulties. It is important to decide on a significant threshold of estimated risk—the threshold at which individuals are considered to be at increased risk. No less important is to decide on an appropriate message, who should convey the message and how. The implications and practical consequences of notifying a group of people—most of whom are not expected to become ill—that they are at increased risk for vCJD must be carefully considered. Authorities should balance the public health goals of a notification against the potential for causing individual harm—not the least of which is anxiety—imposed on individuals and their families, particularly because there is no practical action available to identify those persons who were actually infected or to help them avert illness.

The risk of secondary transmission of vCJD through blood, blood components and plasma derivatives depends upon the prevalence of vCJD in the donor population, both clinical cases and inapparent or pre-clinical infections, within a country. To consider appropriate measures to protect human and animal health, national authorities should seek two kinds of information regarding the risk of vCJD:

- (a) Prevalence of BSE infection in native and imported cattle populations and potential BSE agent contamination of products (“internal” and “external” risk factors described below)
- (b) Potential human exposures to the BSE agent

The minimal infectious doses of the BSE agent for humans exposed by various routes (the oral route presumed to be most common) are not known, and not every exposure is expected to result in infection. However, all potential opportunities for human exposure should be minimized. The likelihood of human exposure to BSE depends upon internal and external factors:

- Internal factors. The internal or national risk of exposure depends on the geographical risk of BSE infections in cattle and the domestic patterns of preparing and using bovine-derived products: (i) cattle feeding practices—especially intentional or accidental use of feeds containing ruminant-derived non-milk proteins like meat-and-bone meal; (ii) slaughter practices, including age of cattle at slaughter, stunning techniques, removal of high-risk tissues from carcasses, containment and disposal of high-risk tissues; and (iii) the national use and distribution of meat and meat products.
- External factors. External risk is the potential exposure of humans to the BSE agent through the importation of infected animals or contaminated animal products and through exposure

of persons traveling outside the country in geographical areas where BSE is present in native or imported beef products and where appropriate risk-reducing controls have not been implemented.

The joint meeting of WHO/FAO/OIE in 2001 (<http://www.who.int/zoonoses/diseases/en>) encouraged all countries to evaluate their potential exposure to BSE through systematic assessment of trade data and other possible risk factors. Such assessments are essential to identify and address risks to protect public health and prevent further spread of BSE.

In most cases the likelihood of vCJD in a country that has no BSE in cattle depends upon the extent to which people were either exposed outside the country or consumed imported commodities, such as beef products and by-products, contaminated with BSE agent. It is clear that countries without BSE in native cattle can still have cases of vCJD. Countries must be prepared to investigate vCJD cases with careful attention to possible internal and external exposures to the BSE agent.

Evidence from studies of blood from infected animals plus recent limited but convincing evidence from human case reports, all indicate that blood transfusion efficiently transmits some TSEs, including vCJD. Leukoreduction is known to remove some TSE infectivity from endogenously infected blood and might be expected to lower risk from labile blood components for transfusion without abolishing risk completely. Leukoreduction is already used widely in some countries, largely for other reasons. Unfortunately, recent studies found substantial endogenous infectivity remaining in plasma after blood of scrapie-infected hamsters was passed through a commercial leukoreduction filter.

For plasma-derived products, the major factors that influence overall risk—as estimated by sensitivity analysis in vCJD risk assessments—include the prevalence of vCJD, the estimated number of vCJD donations per plasma pool, and possible reduction of the TSE infectivity provided by some steps during manufacture. There is a caveat for interpretation of investigational TSE clearance studies: because the natural physical form of the infectious TSE agent in blood is not known, the relevance of studies spiking various materials derived from infected brain tissue into blood that is then processed by scaled-down manufacturing steps remains unclear. Nevertheless, some national regulatory authorities have accepted the results of such studies—with all their uncertainties—as providing assurance that processes used to fractionate plasma are likely to reduce the risk from certain plasma derivatives. That conclusion has been reinforced by epidemiological studies in the UK, where, so far, no case of vCJD has been identified in any recipient of plasma derivatives.

## Risk-reducing measures

### ***Product retrieval and market withdrawal***

Cases of vCJD have been reported in persons who previously donated blood. When such cases are identified, measures must be considered to reduce the risk of person-to-person transmission of infection, including the retrieval of in-date blood and blood components including plasma, as well as pools of plasma and manufacturing intermediates containing the donation. As a precaution, plasma derivatives prepared from those pools should also be identified and withdrawn from the market. In countries with good systems for tracing recipients of blood components from donors later diagnosed with vCJD, the recipients should be notified in an appropriate fashion and enrolled in follow-up surveillance. When a risk assessment indicates that the general population has been potentially exposed to BSE agent through food, additional actions to minimize risks should be considered, varying according to the probable prevalence of vCJD infections in the country.

### ***Donor deferral***

In countries where the risks of BSE in cattle and imports of commodities contaminated with BSE agent are both considered to be low or minimal, another possible source of vCJD infection remains—travel outside the country to areas where the BSE agent might have been present in meat products. In view of the probable long incubation periods of vCJD after oral exposure to the BSE agent—in excess of ten years—risk assessments must recognize that the travel to a country with

BSE may have taken place many years earlier. Some countries have addressed this risk by deferring blood donors with a past history of travel or residence in specified areas for defined cumulative exposure periods. The general consensus is that even in the UK humans were probably not exposed to the BSE agent before 1980. Opportunities for human exposure were markedly diminished by a series of food-protective measures introduced in the UK progressively from June 1988 and fully implemented there by the end of 1996. Other countries subsequently implemented similar measures. Policies for deferring certain blood and plasma donors were adopted in some countries based on surveys of travel histories of repeat blood donors, considering the relative risk reduction afforded by various sets of criteria for donor suitability and estimating the shortages of blood products to be expected. The decision to defer donors with a history of foreign travel or residence, as a precaution to reduce the risk of transmitting vCJD by blood components and plasma derivatives, should be taken by a country after conducting a risk assessment and risk-benefit analysis.

### ***Deferral of transfusion recipients as blood donors***

Mathematical modeling indicated that recycling of vCJD infection via blood transfusion might significantly increase the number of infected persons in the UK. As noted above, all transfusion recipients have therefore been deferred as blood donors in the UK and other countries. The advisability of introducing such policies in response to the risk of vCJD depends on several factors but especially on the estimated prevalence of infection in the donor population. As a less drastic precautionary measure, some countries have deferred only those donors who received blood transfusions in the UK. This decision was based on the new information from the UK showing that the risk of transfusion-transmitted vCJD can no longer be considered simply theoretical. Recently, some countries are proposing to extend this deferral to donors transfused in France.

### ***Plasma products***

To date, there is no evidence that vCJD has been transmitted by human plasma derivatives, even though, at least in the UK, some patients incubating vCJD have contributed to the pools of plasma from which derivatives were manufactured. Several analyses of the risk from plasma derivatives have been conducted, yielding somewhat discordant results, mainly resulting from differences in key assumptions used for the risk models. Several steps in the processes used to manufacture plasma derivatives are likely to reduce the amounts of infectivity, if any, in end products. As noted above, measures to minimize the risk from plasma derivatives have been implemented in the UK, the country reporting the largest number of vCJD cases and highest estimated prevalence of infected persons.

### ***Appropriate blood usage***

As noted by previous WHO Consultations, one simple and especially effective way to reduce the risk of transfusion-transmitted infections, including vCJD, is to adopt a very conservative approach to the utilization of blood, blood components and plasma derivatives, by eliminating their inappropriate prescription. UK authorities recently reported a substantial reduction in use of red cell concentrates without observing any apparent adverse effect on patient outcome.

### ***Other measures***

In countries with a high estimated prevalence of vCJD infection, additional precautionary measures may be considered including importation of specific blood components from low-risk areas to treat especially vulnerable populations and importation of plasma to manufacture derived medicinal products.

The eventual impact of human exposures to BSE agent and secondary transmissions of vCJD via blood, blood components and plasma-derived products cannot be predicted. Countries should not become complacent about their risk from BSE. The extremely low initial national incidence and the low within-herd incidence of BSE cases, long incubation periods and non-specific nature of the early clinical signs of BSE often delay detection of the first cases and mask the sever-

ity of the problem. CJD and vCJD are rare diseases that can be reliably identified only through systematic surveillance. International risk management strategies should be commensurate with the estimated levels of BSE and vCJD risks for individual regions and countries. When selecting appropriate specific risk management strategies, each country must consider the feasibility of implementation—with special attention to maintaining an adequate supply of blood components and plasma derivatives—and should provide a means to audit compliance.

### **Future developments**

- (a) Continuing research into the risk of blood transfusion for transmitting vCJD remains essential. Further information is required on the relative risk for blood recipients, the range of incubation periods of vCJD after food-borne exposures, the period during which blood of an infected donor is infectious prior to onset of clinical signs of vCJD, the spectrum of clinical illness and histopathological findings in transfusion-transmitted vCJD, and possible novel strain characteristics of the blood-borne infectious agent.
- (b) Continuing systematic surveillance for vCJD is necessary in order to identify the risks from blood products and plasma derivatives obtained from donors who subsequently develop vCJD. The UK appendix and tonsil study, while important, cannot be used to estimate prevalence of vCJD infections in other countries of the EU or in other regions.
- (c) Epidemiological studies in other human TSEs, especially sCJD, should be encouraged. Transfusion transmission of sCJD has not been recognized, but several countries long ago implemented precautionary policies deferring certain donors at increased risk for iatrogenic or familial CJD, based on studies in animals showing infectivity in blood during incubation periods of TSEs as well as during overt illness.
- (d) Research should continue to investigate the efficiency of manufacturing processes in reducing infectivity in blood, blood components and plasma-derived products and in developing new techniques to achieve this aim. Such studies should include animal models—in sheep, rodents and possibly non-human primates—with endogenous TSE infections of blood in addition to studies using a variety of spiked materials extracted from brains and spleens of experimentally infected animals.
- (e) Research should also continue to develop and validate new decontamination procedures to lower the risks of cross-contaminations for materials and devices which are not compatible with WHO-recommended procedures.
- (f) 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.

### **The risk of transmitting vCJD by human cells, tissues and tissue derived products**

In addition to transmission of TSE through blood products, transmission by human tissues is also possible, either because of intrinsic infectivity of the tissue or due to contamination with residual blood. In principle, although the same precautions should be applied to the transfer of cells and tissues as to blood, the assessment of TSE transmission risk versus therapeutic benefit should determine the use of such products. For example, the need for a rare type of bone marrow matched only by a UK resident donor would outweigh the remote possibility of TSE transmission. Such a policy might not be appropriate for a more readily available human tissue like cornea that need not be antigenically matched to the recipient. Special consideration should address safety of bone-derived materials widely used in dentistry and orthopedic surgery, which, like cornea, need not be matched. Thus, the risk-benefit estimates for therapeutic administration of products derived from human cells and tissues are probably best decided case-by-case.

# CONCLUSIONS

The potential risks for a given medicinal product administered to humans should be considered case-by-case, taking into account all the foregoing factors and the potential benefits to patients. Recommendations in these Guidelines are intended to apply to all medicinal products for which active substances, excipients or reagents derived from bovine tissues are used during their production processes. The recommendations relate particularly to materials of bovine origin, but the same principles should also be applied to materials used in the manufacture of medicinal products when these are obtained from sheep, goats and other species naturally affected with TSEs. The development of substitutes for bovine, ovine or caprine materials used to manufacture medicinal products is encouraged. This approach may not be feasible for some products, but the goal is sufficiently important to justify long-term attempts to reach it.

It is emphasized again that, when considering precautionary measures to maintain the safety of blood products, authorities should take into account their possible effect on the supply of blood. In that regard, it seems premature to recommend any global uniform policy. Every country should conduct its own analysis to decide if precautionary measures are indicated to reduce the risk of transmitting CJD and vCJD by blood products.

Participants felt it important to stress that eliminating inappropriate use of blood and blood products would substantially reduce the risk of transfusion-related adverse events including the potential risk of blood-borne transmission of TSEs. Development of reliable methods to remove or inactivate the TSE agents during the processing of blood and plasma remain of paramount importance and are to be encouraged, as are efforts to develop and validate sensitive and specific antemortem TSE diagnostic tests suitable for blood donor screening and for qualifying donor units. Similar considerations apply to other human tissue derived products.

## Annex 1

# MAJOR CATEGORIES OF INFECTIVITY: TABLES IA, IB, IC

The information in these Tables is based exclusively upon observations of naturally occurring disease, or primary experimental infection by the oral route (in ruminants), and does not include data on models using strains of TSE adapted to experimental animals, because passaged strain phenotypes can differ significantly and unpredictably from those of naturally occurring disease. Also, because the detection of misfolded host prion protein (PrP<sup>TSE</sup>) has proven to be a reliable indicator of infectivity, PrP<sup>TSE</sup> testing results have been presented in parallel with bioassay data. Tissues are grouped into three major infectivity categories, irrespective of the stage of disease:

- IA: **High-infectivity tissues:** Central Nervous System (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS.
- IB: **Lower-infectivity tissues:** peripheral tissues that have tested positive for infectivity and/or PrP<sup>TSE</sup> in at least one form of TSE.
- IC: **Tissues with no detectable infectivity:** tissues that have been examined for infectivity and/or PrP<sup>TSE</sup> with negative results.

Data entries are shown as follows:

- + Presence of infectivity or PrP<sup>TSE</sup>
- Absence of detectable infectivity or PrP<sup>TSE</sup>
- NT Not tested
- NA Not applicable
- ? Controversial results
- ( ) Limited or preliminary data

The placement of a given tissue in one or another category can be disease-specific and subject to revision as new data accumulate from increasingly sensitive tests. In fact, it is conceivable that the detection of infectivity using transgenic mice that over-express genes encoding various prion proteins, or the detection of PrP<sup>TSE</sup> using some newly developed amplification methods, might prove to be more sensitive than transmission studies in wild-type bioassay animals, and thus may not correlate with disease transmission in nature.

It is also important to understand that categories of infectivity are not the same as categories of risk, which require consideration not only of the level of infectivity in tissue, but also of the amount of tissue to which a person or animal is exposed, and the route by which infection is transmitted. For example, although the level of tissue infectivity (concentration of infectivity in tissue as reflected by titre) is the most important factor in estimating the risk of transmission by instrument cross-contamination during surgical procedures (e.g., neurosurgery versus general surgery), it is only one determinant of the risk of transmission by blood transfusions, in which a large amount of low-infectivity material is administered directly into the circulation, or the risk of transmission by food that, irrespective of high or low infectivity, involves the comparatively inefficient oral route of infection.

**Table IA: High-infectivity tissues**

CNS tissues that attain a high titre of infectivity in the later stages of TSE and certain tissues anatomically associated with the CNS

Tissues	Human TSEs				Cattle		Sheep & goats	
	vCJD		Other TSEs		BSE		Scrapie	
	Infectivity <sup>1</sup>	PrP <sup>TSE</sup>						
Brain	+	+	+	+	+	+	+	+
Spinal cord	+	+	+	+	+	+	+	+
Retina	NT	+	+	+	+	NT	NT	+
Optic nerve <sup>2</sup>	NT	+	NT	+	+	NT	NT	+
Spinal ganglia	+	+	NT	+	+	NT	NT	+
Trigeminal ganglia	+	+	NT	+	+	NT	NT	+
Pituitary gland <sup>3</sup>	NT	+	+	+	-	NT	+	NT
Dura mater <sup>3</sup>	NT	-	+	-	NT	NT	NT	NT

**Table IB: Lower-infectivity tissues**Peripheral tissues that have tested positive for infectivity and/or PrP<sup>TSE</sup> in at least one form of TSE

Tissues	Human TSEs				Cattle		Sheep & goats	
	vCJD		Other TSEs		BSE		Scrapie	
	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>
<b>Peripheral Nervous system</b>								
Peripheral nerves	+	+	(-)	+	+	+	+	+
Enteric plexuses <sup>4</sup>	NT	+	NT	(-)	NT	+	NT	+
<b>Lymphoreticular tissues</b>								
Spleen	+	+	+	+	-	-	+	+
Lymph nodes	+	+	+	-	-	-	+	+
Tonsil	+	+	NT	-	+	-	+	+
Nictitating membrane	NA	NA	NA	NA	+	-	NT	+
Thymus	NT	+	NT	-	-	NT	+	NT
<b>Alimentary tract</b>								
Esophagus	NT	-	NT	-	-	NT	NT	+
Fore-stomach <sup>5</sup> (ruminants only)	NA	NA	NA	NA	-	NT	NT	+
Stomach/ abomasum <sup>6</sup>	NT	-	NT	NT	-	NT	NT	+
Duodenum	NT	-	NT	NT	-	NT	NT	+
Jejunum <sup>6</sup>	NT	+	NT	-	-	NT	NT	+
Ileum <sup>6,7</sup>	NT	+	NT	-	+	+	+	+
Appendix	-	+	NT	-	NA	NA	NA	NA
Large intestine <sup>6</sup>	+	+	NT	-	-	NT	+	+
<b>Reproductive tissues</b>								
Placenta <sup>8</sup>	NT	-	(+)	-	-	NT	+	+
<b>Other tissues</b>								
Lung	NT	-	+	-	-	NT	-	-
Liver	NT	-	+	-	-	NT	+	NT
Kidney	NT	-	+	-	-	-	-	-
Adrenal	NT	+	-	-	NT	NT	+	NT
Pancreas	NT	-	NT	-	-	NT	+	NT
Bone marrow	-	-	(-)	-	(+)	NT	+	NT
Skeletal muscle <sup>9</sup>	NT	+	(-)	+	(+)	NT	-	+
Tongue <sup>10</sup>	NT	-	NT	-	-	NT	NT	+
Blood vessels	NT	+	NT	+	-	NT	NT	+
Nasal mucosa <sup>11</sup>	NT	NT	NT	+	-	NT	+	+
Salivary gland	NT	-	NT	NT	-	NT	+	NT
Cornea <sup>12</sup>	NT	-	+	-	NT	NT	NT	NT
<b>Body fluids</b>								
CSF	-	-	+	-	-	NT	+	NT
Blood <sup>13</sup>	+	?	-	?	-	?	+	?

**Table IC: Tissues with no detectable infectivity**

 Tissues that have been examined for infectivity and/or PrP<sup>TSE</sup> with negative results

Tissues	Human TSEs				Cattle		Sheep & goats	
	vCJD		Other TSEs		BSE		Scrapie	
	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>	Infectivity	PrP <sup>TSE</sup>
<b>Reproductive tissues</b>								
Testis	NT	-	(-)	-	-	NT	-	NT
Prostate/ Epididymis/ Seminal vesicle	NT	-	(-)	-	-	NT	-	NT
Semen	NT	-	(-)	-	-	NT	NT	NT
Ovary	NT	-	NT	-	-	NT	-	NT
Uterus (non-gravid)	NT	-	NT	-	-	NT	-	NT
Placenta fluids	NT	NT	(-)	NT	-	NT	NT	NT
Fetus <sup>14</sup>	NT	NT	NT	NT	-	NT	-	-
Embryos <sup>14</sup>	NT	NT	NT	NT	-	NT	?	NT
<b>Musculo-skeletal tissues</b>								
Bone	NT	NT	NT	NT	-	NT	NT	NT
Heart/pericardium	NT	-	-	-	-	NT	-	NT
Tendon	NT	NT	NT	NT	-	NT	NT	NT
<b>Other tissues</b>								
Gingival tissue	NT	-	-	-	NT	NT	NT	NT
Dental pulp	NT	-	NT	-	NT	NT	NT	NT
Trachea	NT	-	NT	-	-	NT	NT	NT
Skin	NT	-	NT	-	-	NT	-	NT
Adipose tissue	NT	-	(-)	-	-	NT	NT	NT
Thyroid gland	NT	-	(-)	-	NT	NT	-	NT
Mammary gland/ udder	NT	NT	NT	NT	-	NT	-	NT
<b>Body fluids, secretions and excretions</b>								
Milk <sup>15</sup>	NT	NT	(-)	NT	-	-	-	NT
Colostrum <sup>16</sup>	NT	NT	(-)	NT	(-)	-	-	NT
Cord blood <sup>17</sup>	NT	NT	(-)	NT	-	NT	NT	NT
Saliva	NT	-	-	NT	NT	NT	-	NT
Sweat	NT	NT	-	NT	NT	NT	NT	NT
Tears	NT	NT	-	NT	NT	NT	NT	NT
Nasal mucus	NT	-	-	NT	NT	NT	NT	NT
Bile	NT	NT	NT	NT	NT	NT	NT	NT
Urine <sup>16,17</sup>	NT	NT	-	-	-	NT	NT	NT
Feces	NT	NT	-	NT	-	NT	-	NT

## Annex 1 Footnotes

1. Infectivity bioassays of human tissues have been conducted in either primates or mice (or both); bioassays of cattle tissues have been conducted in either cattle or mice (or both); and most bioassays of sheep and/or goat tissues have been conducted only in mice. In regard to sheep and goats, not all results are consistent for both species.
2. In experimental models of TSE, the optic nerve has been shown to be a route of neuroinvasion and contains high titres of infectivity.
3. No experimental data about infectivity in human pituitary gland or dura mater have been reported, but cadaveric dura mater allograft patches, and growth hormone derived from cadaveric pituitaries have transmitted disease to hundreds of people and therefore must be included in the category of high-risk tissues.
4. In cattle, PrP<sup>TSE</sup> was limited to enteric plexus in the distal ileum.
5. Ruminant fore-stomachs (reticulum, rumen, and omasum) are widely consumed, as is the true stomach (abomasum). The abomasum of cattle (and sometimes sheep) is also a source of rennet.
6. In vCJD, transmission to mice has so far been limited to rectal tissue, and PrP<sup>TSE</sup> was detected only in gut-associated lymphoid and nervous tissue (mucosa, muscle, and serosa were negative). In goats, PrP<sup>TSE</sup> was also limited to gut-associated lymphoid and nervous tissue [Andreoletti, unpublished data]
7. In cattle and sheep, only the distal ileum has been bioassayed for infectivity.
8. A single report of transmission of CJD infectivity from human placenta has never been confirmed and is considered improbable.
9. Muscle homogenates have not transmitted disease to primates from humans with sCJD, or to cattle from cattle with BSE. However, intracerebral inoculation of a semitendinosus muscle homogenate (including nervous and lymphatic elements) from a single cow with BSE has transmitted disease to PrP over-expressing transgenic mice at a rate indicative of only trace levels of infectivity. Also, recent published and unpublished studies have reported the presence of PrP<sup>TSE</sup> in skeletal muscle in experimental rodent models of scrapie and vCJD, in experimental and natural infections of sheep and goats, in sheep orally dosed with BSE [Andreoletti, unpublished data], and in humans with sCJD, iCJD and vCJD. Bioassays to determine whether PrP<sup>TSE</sup> is associated with transmissibility in these experimental or natural infections are in progress.
10. In cattle, infectivity bioassay was negative, but the presence of PrP<sup>TSE</sup> in palatine tonsil has raised concern about possible infectivity in lingual tonsillar tissue at the base of the tongue that may not be removed at slaughter.
11. In sCJD, PrP<sup>TSE</sup> is limited to olfactory mucosa.
12. Because only one or two cases of CJD have been plausibly attributed to corneal transplants among hundreds of thousands of recipients, cornea is categorised as a lower-risk tissue; other anterior chamber tissues (lens, aqueous humor, iris, conjunctiva) have been tested with a negative result both in vCJD and other human TSEs, and there is no epidemiological evidence that they have been associated with iatrogenic disease transmission.
13. A wealth of data from studies of blood infectivity in experimental animal models of TSE has been extended by recent studies documenting infectivity in the blood of sheep with naturally occurring scrapie, and, from epidemiological observations, three blood-associated vCJD transmissions in humans. Blood has not been shown to transmit disease from patients with any other form of TSE, or from cattle with BSE, including fetal calf blood. However, several laboratories using new, highly sensitive methods to detect PrP<sup>TSE</sup> claim success in studies of plasma and/or buffy coat in a variety of animal and human TSEs. Because the tests are all in a preliminary stage of development, and do not yet include results on blinded testing of specimens from naturally infected humans or animals, the Consultation felt that it was still too early to evaluate the validity of these tests with sufficient confidence to permit either a negative or positive conclusion.
14. Embryos from BSE-affected cattle have not transmitted disease to mice, but no infectivity measurements have been made with fetal calf tissues other than blood (negative mouse bioassay). Calves born of dams that received embryos from BSE-affected cattle have survived for observation periods of up to seven years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy or PrP<sup>TSE</sup>.
15. Evidence that infectivity is not present in milk includes temporo-spatial epidemiologic observations failing to detect maternal transmission; clinical observations of over a hundred calves nursed by infected

cows that have not developed BSE; and experimental observations that milk from infected cows has not transmitted disease when administered intracerebrally or orally to mice. Also, PrP<sup>TSE</sup> has not been detected in milk from cattle incubating BSE following experimental oral challenge.

16. Early reports of transmission of CJD infectivity from human cord blood, colostrum, and urine have never been confirmed and are considered improbable. A recent bioassay in PrP over-expressing transgenic mice of colostrum from a cow with BSE gave a negative result; and PrP<sup>TSE</sup> has not been detected in colostrum from cattle incubating BSE following experimental oral challenge.
17. IgG short chains mimicking the Western blot behavior of PrP<sup>TSE</sup> have been identified in the urine of sporadic, variant, and familial CJD patients.

## Table references

Tables IA, IB and IC are an update from tables created in an earlier Consultation (WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products, 2003, <http://www.who.int/bloodproducts/tse/en>) by an ad hoc expert interim group composed of Dr O. Andreoletti, Mr R. Bradley, Dr P. Brown, Prof Dr H. Budka, Prof Dr M.H. Groschup, Prof J.W. Ironside, Prof M. Pocchiari, and Mr G.A.H. Wells. Dr P. Brown coordinated the group and consolidated the information for review by all Consultation participants.

Most of the observations that form the basis for the Tables have been published in original reports (or cited in reviews) that follow. Most studies published since the previous Consultation have been listed, but no attempt has been made to list the many earlier reports in which only one or two tissues were examined unless they concerned tissues of exceptional current interest. Also, a number of observations made by or known to members of the expert group that assembled the Tables have not yet been published.

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## Annex 2

# SUMMARIES OF SCIENTIFIC PRESENTATIONS

## EPIDEMIOLOGY

### Epidemiology update on human TSE diseases

#### *R G Will*

Risk assessments in relation to vCJD are influenced by trends in the incidence of this disease, which is likely to have been caused by transmission of BSE to humans. The number of annual deaths from vCJD in the UK has been in decline since 2000 and projections of the future total numbers of cases in the UK have become more conservative with time. Similar analyses in Ireland and France have suggested limited future epidemics. However, there is evidence from a case-control study that vCJD was probably caused by dietary exposure to high-titre bovine tissue in the food chain (Ward, Everington et al. 2006); human exposure to BSE was probably extensive in the UK but also took place in other countries. Single cases of vCJD have recently been identified in a number of previously unaffected countries and the possibility of this condition occurring in further countries cannot be excluded. In view of the probable secondary transmission of vCJD through blood transfusion, it is important for all countries to consider the risk of BSE and vCJD in their populations (Chadeau-Hyam and Alperovitch, 2005; Clarke and Ghani, 2005; Will, 2005).

### Epidemiology update on animal TSE diseases

#### *R Bradley*

In regard to animal TSEs other than BSE, scrapie still occurs in many countries. Transmissible mink encephalopathy (TME) has not been reported since the 1980s. Chronic wasting disease (CWD) has a wider recognized geographical range in North America than before; an infected animal was accidentally exported to the Republic of Korea (Sohn, Kim et al. 2002). CWD has not been reported in Europe (EC, 2005a).

In regard to BSE-related diseases, BSE is declining in Europe and the mean age of onset in cattle has increased (EC, 2005b). No cases of feline spongiform encephalopathy (FSE) in domestic cats have been reported since 2001; the last case of FSE in a captive wild cat was in an animal born in 1992 and the last case of spongiform encephalopathy in captive wild Bovidae was recognized in 1993 (DEFRA, 2006). Since December 2002, seven cases of BSE in native-born cattle have been reported for the first time in North America, five in Canada and two in the USA (OIE, 2006). One case of BSE was confirmed in a domestic goat in France (Eurosurveillance, 2005) and another possible historical case (1990) has been reported in Scotland.

The source of BSE in cattle is not known and may never be known with certainty. Meat-and-bone meal has been the major, if not the only, vehicle of transmission to ruminants (DEFRA, 2005). Paternal and horizontal transmissions are not factors in the transmission of BSE and no evidence supports maternal transmission in the absence of a feed-borne source. In Europe, specified-risk materials (SRM) are completely destroyed. The OIE (OIE, 2005) recommends that ruminant protein derived by rendering should be pressure cooked. In North America, SRM are removed from human food but if rendered they are not pressure cooked and may currently be fed to non-ruminant animals. The OIE has determined three categories of geographical BSE risk: Negligible, Controlled and Undetermined (OIE, 2005). Protein-free tallow may be traded as can other tallow if from a negligible-risk country or from a controlled-risk country if SRM are removed. Chemical derivatives from this tallow for use in pharmaceuticals, biologicals or medical

devices—if produced by hydrolysis, saponification or transesterification using high temperature and pressure—can also be traded. Gelatin and collagen from skins, or from bones from a negligible-risk country, may also be traded. Gelatin from bones from a controlled-BSE-risk country may also be traded, so long as SRM (skulls and vertebrae) are removed followed by pressure washing, acid demineralisation, prolonged alkaline treatment, filtration and heat sterilisation ( $\geq 138^\circ\text{C}$  for  $\geq 4$  sec) or an equivalent process (OIE, 2005). Over 11 million rapid tests for BSE have been conducted in the EU annually in recent years (EC, 2005a). In 2004, 664 cases (79% of the total) were detected in the original EU 15 countries by active surveillance and 175 cases (21%) by passive surveillance; in the same year, 27 BSE cases were found in the ten new countries of the EU (the “New 10”), all by active surveillance (EC, 2005a).

Well enforced feed bans and SRM bans have been responsible for the decline in BSE in Europe. In 2004 in Great Britain the age of peak incidence of BSE in cattle had increased to ten years and in the rest of the EU to six years (EC, 2005a). In the EU 25 in 2004,  $\geq 2,600$  cases of scrapie were detected in sheep and about 400 in goats, mostly by active surveillance; Cyprus reported the largest number of scrapie cases, 1,208 in sheep and 354 in goats (EC, 2005a). One case of BSE in a French goat has been confirmed by biological strain typing (Eurosurveillance, 2005). TSE testing of samples from domestic cats, other carnivores, pigs, horses and deer have all proved negative (EC, 2005a). In the light of an improving situation in the EU (fewer cases year on year and older age at onset), the EC has discussed with Member States and the European Parliament possible amendments to the current BSE measures that will still eliminate BSE without endangering the consumer (EC, 2005b). The cost of BSE monitoring in the EU from 2001–2004 has been 1,612 million € and the cost per detected case 1.56 million € (EC, 2005b). It is proposed to reduce these costs by modifying measures in the short and long term as outlined in an EC “TSE Roadmap” (EC, 2005b). This analysis marks the beginning of the end for BSE in the EU and Switzerland and shows that targeted active surveillance and rapid testing, especially of fallen stock and emergency slaughter animals, play an increasingly dominant role in BSE surveillance. TME, FSE and zoo animal TSE have become historical diseases. CWD is unreported in Europe but few tests have been done. Scrapie still exists in Europe and other places; it would be premature to predict its decline.

## Update on vCJD prevalence estimated from tonsil and appendix screening

### *J W Ironside*

Lymphoid tissues are more involved in variant CJD than in other forms of the disease. PrP<sup>TSE</sup> has been readily detected in lymphoid tissues in the body by immunoblotting and immunohistochemistry (Ironside, Head et al. 2000). Accumulations of PrP<sup>TSE</sup> in lymphoid tissues are associated with infectivity, at levels around 100 times lower than those in the brain (Bruce, McConnell et al. 2001). PrP<sup>TSE</sup> was detected in two appendixes, one removed around eight months and the other two years prior to onset of clinical vCJD, but not in a third removed nine years before onset (Hilton, Fathers et al. 1998; Hilton, Ghani et al. 2004).

Despite ethical constraints on the study, the prevalence of vCJD infection was estimated by a retrospective search for PrP<sup>TSE</sup> accumulation in archived appendix and tonsil tissues in UK. The immunohistochemistry test used was very unlikely to have been 100% sensitive but it appeared to be specific (Hilton, Sutak et al. 2004). PrP<sup>TSE</sup> was detected in three of 12,674 appendix and tonsil samples. All positive samples were appendixes (Hilton, Ghani et al. 2004). Based on that study, the prevalence of vCJD infection was estimated at 237/million (95% CI 49–692/million) in the general UK population (not age-adjusted). Two of the positive samples had a pattern of PrP accumulation in germinal centres somewhat different from that seen in lymphoid tissues of confirmed vCJD cases; genetic analysis of DNA extracted from these 2 cases found both to have been from persons homozygous for valine at codon 129 of the prion-protein-encoding (PRNP) gene (Ironside, Bishop et al. 2006). This finding indicates that individuals homozygous for valine at PRNP codon 129 are susceptible to infection with the BSE agent, although, thus far, all clinical cases of vCJD have been in persons homozygous for methionine at that locus. The second presumptive transfusion-transmitted vCJD infection was observed in a neurologically normal person

heterozygous for methionine and valine at PRNP codon 129 (Peden, Head et al. 2004). Whether persons with PRNP-129 methionine-valine-heterozygous and valine-homozygous genotypes are susceptible to clinical vCJD—perhaps after longer incubation periods than those of methionine-homozygous individuals—remains uncertain. A larger prospective study involving about 100,000 tonsil specimens is underway in UK using both immunoblotting and immunohistochemistry to detect PrP<sup>TSE</sup>.

### ***J Wadsworth***

In another UK study, no abnormal PrP positive samples were found in 2000 tonsil pairs by either immunoblotting or immunocytochemistry; these negative results provide little reassurance, because half the patients were less than nine years old and so unlikely to have come into contact with BSE-infected material (Frosh, Smith et al. 2004). All tonsil specimens tested from vCJD autopsies were uniformly positive for PrP<sup>TSE</sup>. Formalin fixation must be avoided and fresh frozen tissue obtained if immunoblotting detection of PrP<sup>TSE</sup> or infectivity studies are intended. Conversely PrP<sup>TSE</sup> was detected only irregularly in vCJD appendixes, apparently related to sampling variation due to a lower density of lymphoid follicles. To date, PrP<sup>TSE</sup> concentration has been measured in the appendix of only a single vCJD patient and reported to be 200-fold lower than in brain (Joiner, Linehan et al. 2002). PrP<sup>TSE</sup> was uniformly detected in terminal ileum of vCJD cases in concentrations up to 1% of those in brain (Joiner, Linehan et al. 2005) but only irregularly in rectum at a much lower concentration (0.002% of brain) (Wadsworth, Joiner et al. 2001).

Transmission studies with transgenic mice (homozygous for methionine at codon 129 of PRNP transgene [129MM]) indicate that the ratio of PrP<sup>TSE</sup> to infectivity in tissues from vCJD cases may closely resemble that of experimental prion infections in rodents (Wadsworth, Joiner et al. 2006). These findings endorse the use of PrP<sup>TSE</sup> analysis for vCJD to help devise risk reduction strategies that might limit secondary transmission of vCJD prions.

### ***M Glatzel***

A Swiss tissue bank has also been developed to screen human lymphoid tissue for the presence of PrP<sup>TSE</sup>. So far, 920 samples from surgery and 940 from autopsy have been studied and no positive case found.

## **PROGRESS IN DETECTION AND QUANTITATION OF INFECTIVITY**

### **Bioassays**

#### ***Primates***

##### ***C I Lasmézas***

A cynomolgus macaque model has been used to mimic human infections with the agents of variant, sporadic or iatrogenic CJD. Recent data indicate that 5 g of brain from a clinical BSE case is necessary to infect a primate (Lasmézas, Comoy et al. 2005). The model allows a preliminary assessment of the efficiency of current measures to protect the human food chain. Taking into account the limit of sensitivity for the most sensitive tests used in abattoirs (a dilution of brain 1/300), the combination of specified-risk materials removal and proper testing should be adequate to protect the human food chain and consumer health. Blood transmission experiments with vCJD and BSE agents in this model indicated that the intravenous (IV) route is very efficient and gives an incubation period similar to that after direct intracerebral (IC) inoculation (Herzog, Sales et al. 2004). Moreover, tissue distribution of PrP<sup>TSE</sup> has been similar after primary and secondary transmissions, suggesting that secondary transmission of vCJD may present a risk to similar to that of primary exposure. Ongoing experiments with blood—collected before the onset of clinical illness from monkeys inoculated with vCJD or BSE agents IC or IV—will provide valuable information to assess the risk of secondary vCJD cases in humans linked to transfusion.

## Transgenic mice

### G Telling

Numerous transgenic mouse models have been developed with various human, bovine, ovine and other PrP-encoding genes attempting transmissions of many TSE agent strains. Increased transmissibility, shorter incubation periods, and more sensitive detection of infectivity are several advantages offered by these models. However, while a transmission barrier to vCJD prions apparently does not exist in transgenic mice expressing the bovine PrP-encoding gene—Tg(BoPrP) mice—bioassays of vCJD prions in mice expressing the human PRNP gene—Tg(HuPrP) mice—remains problematic. Moreover, some Tg mice overexpressing PrP develop spontaneous neurological disease late in life, which impairs their use as an assay for small amounts of infectivity (Nazor, Khun et al. 2005). Furthermore, the sensitivities to infection of different Tg mouse lines have not yet been compared by conducting parallel titrations with the same TSE reference materials to standardize the assays.

### PL Gambetti

In North America there has been growing concern about a possible risk to humans exposed to the agent of chronic wasting disease (CWD). The number of deer and elk in the USA is estimated to exceed 22 million captive and free-range animals. A recent program of active surveillance (in which 126,000 tests were conducted during 2002-2003) suggested prevalence of the disease around 5%. Attempts to transmit CWD from brains of infected cervids to Tg mice overexpressing elk PrP succeeded but those to Tg mice overexpressing human PrP did not, suggesting the existence of a substantial species barrier between cervids and humans that might protect humans from infection with the CWD agent (Kong, Huang et al. 2005; Gambetti, unpublished).

## Bank voles

### U Agrimi

A new experimental TSE model has been described in bank voles (*Clethrionomys glareolus*) (Cartoni, Schinina et al. 2005). These rodents are susceptible to a wide range of animal and human TSEs, major exceptions being a low susceptibility to infection with the BSE agent and to the atypical scrapie strain Nor98. The voles exhibited 100% transmission rates with short survival times (150-250 days) after inoculation with most scrapie isolates, type-1 sporadic CJD and CWD. All human and animal TSE agents tested—BSE included—adapted to infect voles with very short survival times (70-150 days). The molecular properties of PrP<sup>TSE</sup> from the TSE isolates tested were maintained in immunoblotting with PrP of infected voles. Voles also appear to be well suited to detect low levels of infectivity in samples. The exceptionally wide host range from which different prion strains were transmitted to voles should allow characterization and comparison of isolates. Strain effects seemed to be more important than host of origin (Nonno, Di Bari et al. 2006).

## In vitro assays/cell cultures

### PC Klöhn

Sensitive *in vitro* assays can be developed by taking advantage of the cell-based amplification of TSE agents. Several cell lines that accumulated PrP<sup>TSE</sup> were infectious. Compared to immunoblotting, which can detect PrP<sup>TSE</sup> in a 10<sup>-5</sup> dilution of infected brain tissue (0.5-5 µg [Wadsworth, Joiner et al. 2001]), some *in vitro* assays exceeded this level of sensitivity by two to three orders of magnitude due to cell-based amplification of the TSE agent. The scrapie cell assay (SCA) was positive in a 10<sup>-8</sup> dilution of a mouse brain suspension containing 10<sup>8.4</sup> mouse IC LD50/g; the SCA method showed sensitivity levels comparable to those of bioassays in mice but was faster, less expensive and offered the possibility of automation (Klöhn, Stoltze et al. 2003). A sensitive slot blot (Winklhofer, Hartl et al. 2001) and a cell colony assay (Bosque and Prusiner, 2000) have also been described. A scrapie cell blot has also been used to screen potential therapeutically active compounds (Kocisko, Baron et al. 2003). To date, the failure to identify cell lines susceptible to human, ovine and bovine prions has impeded developing fast and comprehensive cell-based testing for prion infectivity.

Cell-based assays can give rise to false-positive results due to PK-resistant non-infectious forms of PrP (Vorberg, Raines et al. 2004). However, because of the dynamic nature of the process of prion propagation in cells, true-positive results can be distinguished from false-positive results by comparing consecutive cell passages. Infectivity in a susceptible cell line (N2aPK1) was reported to accrue at a rate of 20%-30% per day (Klöhn, Stoltze et al. 2003); in contrast, the number of false-positive cells detected, for instance in the presence of proteasome inhibitors, decreased progressively as cells proliferated.

### Cautionary artifacts in detection of PrP<sup>TSE</sup>

#### *RG Rohwer*

Other artifacts have been observed in the detection of PrP<sup>TSE</sup>. The PrP amplification system called “protein misfolding cyclical amplification” (PMCA (Castilla, Saa et al. 2005)), relies on multiple cycles of sonication. Vigorous sonication has sometimes formed aggregated PrP<sup>C</sup> that resisted protease treatment but did not show the band shift on immunoblotting to the smaller mass typical of PrP<sup>TSE</sup> (Rohwer, unpublished); in some assays these proteinase K-resistant aggregates might be confused with misfolded PrP. Another situation leading to erroneous identification of PrP<sup>TSE</sup> resulted from accidental detection of immunoglobulin light chains by a secondary detector antibody (Serban, Legname et al. 2004). Finally, because of the extremely high concentrations of TSE agent present in some brain tissues—in which infectivity titres can exceed those in blood by 10<sup>9</sup>-fold—cross contamination of other tissues can easily occur, leading to false positive results.

## TISSUE OR BODY FLUID INFECTIVITY

### TSE infectivity in muscles and peripheral nervous system

#### *CJD patients*

##### *M Glatzel*

In humans, PrP<sup>TSE</sup> accumulations have been observed in muscles of some patients with sporadic CJD (Glatzel, Abela et al. 2003). Long duration of disease predisposes to PrP<sup>TSE</sup> accumulation in muscle and abundant PrP<sup>C</sup> levels in muscle might facilitate PrP<sup>TSE</sup> accumulation (Kovacs, Lindeck-Pozza et al. 2004). In rodents, PrP<sup>TSE</sup> can be detected in muscle both before and after the onset of clinical illness (Beekes, comment). A ten-fold depletion of PrP<sup>C</sup> in skeletal muscle did not alter morphology but abolished PrP<sup>TSE</sup> production in 75% of mice studied (Glatzel, unpublished).

#### *Sheep with scrapie*

##### *O Andreoletti*

PrP<sup>TSE</sup> has been detected before onset of illness in muscles of sheep and goats infected with scrapie by the oral route. After massive oral inoculation of scrapie-sensitive sheep (with PrP phenotype VRQ at codons 136, 154 and 171 respectively) intestine became positive 18 days post inoculation (PI), muscle and nerves between 30 and 90 days PI, and central nervous system at 6 months PI; death occurred 14 months PI. PrP<sup>TSE</sup> accumulation was associated with infectivity estimated to have been equivalent to 10<sup>-3</sup>-10<sup>-4</sup> diluted obex. Distribution of PrP<sup>TSE</sup> in tissues was extremely heterogeneous: PrP<sup>TSE</sup> was detected by immunohistochemistry in muscle spindles and in Schwann cells of nerves. Ongoing experiment in Tg mice will determine the infectivity titres associated with the different tissues. The total amounts of infectivity in muscle seem to be small ((Andreoletti, Simon et al. 2004) and Andreoletti, unpublished). Preliminary data suggest that small amounts of infectivity might also be present in muscles of sheep with the PrP ARR phenotype after infection with BSE agent. The ARR phenotype, designated by single letters denoting the amino acids encoded by the prion-protein-encoding gene at PrP codons 136, 154 and 171, is expressed by sheep homozygous for the ARR genotype; ARR sheep are relatively, though not absolutely, resistant to natural infections with most scrapie strains and to experimental infection with BSE by the oral route (Houston, Goldmann et al. 2003).

## **BSE, including an update on methodology**

### **The UK BSE studies**

#### **GAH Wells**

Concerning BSE in cattle, the first experimental oral exposure study (a pathogenesis study conducted by UK Veterinary Laboratory Agency [VLA]) was initiated in 1991 using 30 bovines, each dosed with 100 g of BSE-contaminated brain titrating  $10^{3.5}$  ID<sub>50</sub>/g<sup>1</sup> in RIII mice. Animals were sequentially killed from two months up to 40 months post-exposure and tissues taken for infectivity assays in RIII or C57B1mice. In another set of experiments, the infectivity of a BSE bovine brainstem pool (from five cases of BSE) has been compared in RIII mice and cattle to evaluate the species barrier. In RIII mice the brainstem pool contained  $10^{3.3}$  mouse intracerebral (IC) and intraperitoneal (IP) ID<sub>50</sub> /g while in Friesian-Holstein cattle the same material titrated  $10^{6.0}$  IC ID<sub>50</sub> /g; thus, the underestimate of infectivity titre of BSE brain tissue titrated across a species barrier in mice was 500 fold ( $2.7 \log_{10}$ ). The limit of detection of infectivity by the cattle assay was approximately  $10^{-1}$  cattle IC ID<sub>50</sub> units or  $10^{-3.7}$  mouse IC+IP ID<sub>50</sub> units/g. Selected cattle tissues from the first experimental oral exposure study were inoculated into assay cattle to increase the sensitivity of detection of infectivity.

In the initial UK pathogenesis study, infectivity was first detected in a pool of distal ileum from three exposed animals six months after exposure—subsequently all of five cattle inoculated IC with this same pool developed BSE between 23 and 30 months post-inoculation—and PrP<sup>TSE</sup> was detected in Peyer's patches. It is noteworthy that PrP<sup>TSE</sup> has not been detected in the ileum of cows with natural cases of BSE. One pool of lymph nodes (retropharyngeal, mesenteric, popliteal and prescapular) and one pool of spleens from five BSE cases were negative on assay in mice and cattle; this finding implies that the titres of the two pools must have been less than 0.1 cattle IC ID<sub>50</sub>/g. One pool of tonsils from three exposed calves of the first experimental oral exposure study at ten months post-inoculation was positive; one of five cattle inoculated IC with this pool developed BSE 45 months post-inoculation. Also, a pool of nictitating membranes (lymphoid tissue from ten clinically suspect cases of BSE, including nine later confirmed cases) was positive (incubation period 31 months) implying an approximate infectious titre of  $10^1$ - $10^2$  cattle IC ID<sub>50</sub>/g (Wells, unpublished). Assays of tissue infectivity from naturally infected clinical cases in RIII mice showed infectivity only in central nervous system ([Foster, Hope et al. 1994] and Wells, unpublished).

A second UK BSE pathogenesis study, again following oral exposure, was initiated in 1997. Three hundred cattle were divided into three groups: each animal in the first group received an oral dose of 100 g of BSE-affected brain, each animal in the second group received 1 g of affected brain, and the remaining hundred cattle were un-dosed controls. Tissues were sampled from six exposed cattle and three unexposed controls killed sequentially at three-month intervals, starting three months post-inoculation and continuing into the early clinical phase of disease. Sampling was concluded at 45 months (100-g-dose group) or at 78 months post-inoculation (1-g-dose group). Further study of the collected material, some in collaboration with Health Canada, is in progress.

Bioassays of infectivity have inherent limitations. Assays conducted in wild-type mice underestimate infectivity due to the species barrier effect. While this problem is overcome when BSE agent is assayed in cattle, the cost and protracted timescale of these assays are prohibitive for routine application. Assays in mice expressing multiple copies of the bovine PrP gene also overcome the species barrier and offer high sensitivity. All assays for TSE infectivity have limitations in sensitivity due to the non-uniform distribution of infectivity in tissues and the consequent variation resulting from focal sampling and small starting volumes of inocula; those problems are compounded when assays are conducted on tissue pools.

<sup>1</sup>ID<sub>50</sub>, 50% infectious dose, is a term preferred by some investigators to LD<sub>50</sub>, 50% lethal dose. The terms are essentially equivalent for TSEs and both are used in this document.

### **The German BSE studies**

#### ***M Groschup***

A third pathogenesis study, in Germany, was performed using BSE brain stem material kindly supplied from the VLA TSE archive, UK. At the end of 2002, 56 calves were dosed orally with 100 g of BSE brain while 18 control calves received normal brain. Animals have been bled and urine and CSF obtained at two-month or four-month intervals. Serial kills of about four BSE-exposed and one control animal each were performed every four months. More than 150 body tissues and fluids were collected during each necropsy from the sequentially sacrificed animals. Conventional immunohistochemistry and immunoblotting were used to detect abnormal PrP, and Tg(bov) mice overexpressing bovine PrP<sup>C</sup> were inoculated to detect BSE infectivity in these necropsy samples. The sensitivity of the Tg(bov)-mouse bioassay model was verified by comparative infectivity titrations of a single BSE brain stem pool in both conventional wild-type RIII mice and in the Tg(bov) mice; infectivity titres of the BSE pool were 10<sup>7.7</sup> IC LD<sub>50</sub> in Tg(bov) mice and 10<sup>3.3</sup> in RIII mice, i.e., assay in Tg(bov) mice was more than 10,000-fold more sensitive for detecting BSE agent than was assay in RIII mice and ten-fold more sensitive than by that reported for cattle inoculated with BSE agent IC (Buschmann and Groschup, 2005).

The German BSE pathogenesis studies also included an investigation of a ninth German BSE case—a late-stage pregnant cow with naturally acquired BSE. Tissues of that animal were assayed in RIII and Tg(bov) mice; no infectivity was detected in either spleen or lymph nodes, and all samples from the reproductive tract were also free of detectable infectivity. Conversely, infectivity was detected in the Peyer's Patches of the distal ileum by assay with Tg(bov) mice (mean incubation time 540 days) while the RIII mice remained negative even after a subpassage. Infectivity was also found in peripheral nerves (but not radial nerve) assayed in Tg(bov) mice, with incubation times of 438 and 538 days after inoculations with sciatic and facial nerves respectively. It is also noteworthy that one of ten Tg(bov) mice inoculated with one specific muscle (semitendinosus) was positive 520 days post-inoculation (Buschmann and Groschup, 2005).

Atypical BSE cases have now been reported among animals screening positive during systematic testing in Europe of cattle over 30 months old; the cases have been described in Italy (Casalone, Zanusso et al. 2004), France (Biacabe, Laplanche et al. 2004), Denmark, Germany, the Netherlands, and Japan. Most cases have been characterized only by biochemical features of the PrP<sup>TSE</sup>. Only for the two cases reported from Italy is detailed information available describing the light microscopic pathology of the brain, which showed widespread PrP<sup>TSE</sup> in the cerebral cortex with amyloid plaques. These cases—in animals older than most of those with typical BSE—have been called bovine amyloidotic spongiform encephalopathy (BASE). The Japanese atypical BSE cases, conversely, were reported in very young animals (20 and 23 months old), also detected by rapid tests at the slaughterhouse. The origin and pathogenesis of these atypical BSE cases and whether they represent infections with a TSE agent strain different from that causing typical BSE remain unknown.

In sheep, atypical scrapie cases, first identified in Norway and associated with a strain of agent termed Nor98, have now been detected in Belgium, Finland, France, Germany, Ireland, Portugal, Sweden, Switzerland, and the UK. They represent approximately 50 % of all currently diagnosed scrapie cases and 90 % of affected flocks in Germany. Because only one of the rapid tests currently available detects PrP<sup>TSE</sup> in the obex of sheep with atypical scrapie sampled in the slaughterhouse, the number of recognized atypical scrapie cases varies in countries using different tests. Compared to sheep with classical scrapie or even to cows with BSE, sheep with atypical scrapie had a relatively low concentration of PrP<sup>TSE</sup> with a low proteinase K resistance in the obex, which explains why several techniques failed to detect them. The PrP<sup>TSE</sup> from cases of atypical scrapie also had a distinct pattern on immunoblotting. There has been a lower prevalence of overt illness in herds with atypical scrapie compared with herds having classical scrapie. Sheep selected for their resistance to classical scrapie (having prion protein phenotype ARR) were sensitive to experimental infection with these atypical isolates, and natural cases of atypical scrapie have been observed in ARR sheep (Buschmann, Biacabe et al. 2004). Atypical scrapie isolates have been transmitted to transgenic mice expressing ovine PrP.

In France, a BSE case has been reported in a normal slaughter goat born in 2000, i.e., before the full implementation in 2001 of a ban on feeding meat-and-bone meal to ruminants. This case was identified as a TSE in 2002, during the course of an active surveillance program using rapid tests for PrP<sup>TSE</sup>. None of 200 other goats from the same herd was positive at slaughter. The TSE agent from the goat was later investigated by molecular characterization of PrP<sup>TSE</sup> in several laboratories and by determining its histopathological lesion profiles in lines of experimentally infected mice; it has been indistinguishable from a BSE strain. Based on a retrospective analysis of archived tissue samples in Scotland, an even earlier caprine BSE case has been suspected.

### **Experimental models of TSE diseases**

#### **Rodents**

##### **M Beekes**

In the 263K hamster scrapie model, the vagus nerve appears to offer a direct pathway for cerebral infection after invasion of the enteric nervous system following oral exposure. Infection also spreads to the spinal cord via the sympathetic nervous system innervating the digestive tract (McBride, Schulz-Schaeffer et al. 2001). PrP<sup>TSE</sup> was detected in muscle at late stages of preclinical incubation and during clinical illness. Infection of muscles appeared to result predominantly from a centrifugal spread of agent from the central nervous system via efferent nerves. It is estimated that ~10 g of muscle tissue can infect one hamster via the oral route ( $10^2$ - $10^4$  IC LD50 per g of muscle tissue versus  $1$ - $3 \times 10^9$  IC LD50 per g of brain tissue at the terminal stage of scrapie in the 263K hamster model). In hamsters with 263K scrapie infections, PrP<sup>TSE</sup> was detected in muscle under cell membranes of myocytes and scattered within muscle cells; the same pattern of PrP<sup>TSE</sup> deposition also occurred in muscles of hamsters infected intracerebrally with hamster-adapted BSE agent. In hamsters perorally infected with scrapie, PrP<sup>TSE</sup> was observed in structures reminiscent of neuromuscular junctions. The distribution of PrP<sup>TSE</sup>-positive myocytes in muscles exhibited a pattern resembling that of motor units. PrP<sup>TSE</sup> was also detected in muscle spindles, although it was more difficult to find the protein in that location. Taken together, the intramuscular location and distribution pattern of PrP<sup>TSE</sup>, with the protein's late appearance in muscles, are highly suggestive of a centrifugal spread of infection from spinal or cranial motor neurons via their axonal projections to neuromuscular junctions and from there post-synaptically into muscle fibres. However, invasion of muscles might occur via sensory nerve fibers as well (Thomzig, Kratzel et al. 2003; Thomzig, Schulz-Schaeffer et al. 2004; Thomzig, Cardone et al. 2006).

##### **RA Bessen**

PrP<sup>TSE</sup> was detected in tongue and nasal turbinates of scrapie-infected sheep during clinical illness. In hamsters infected IC with the HY strain of TME agent, PrP<sup>TSE</sup> appeared in the musculature and epithelium of the tongue; prion infection was found in skeletal muscle cells, taste cells, and nerve fibers (and possibly in epithelial cells) of the tongue as well as in the olfactory epithelium of the nasal cavity. This distribution of the HY TME agent was consistent with prion spread from brain stem to peripheral tissues via cranial nerves, specifically via the hypoglossal nerve to the skeletal muscle in the tongue, via the chorda tympani to the taste cells in the tongue, via the lingual nerve to the nerve fibers in epithelium of the tongue, and via olfactory nerve to sensory neurons in the olfactory epithelium. This centrifugal spread of the HY TME agent to the oral and nasal mucosa might be involved in shedding of prions and horizontal transmission of infection. The tongue also served as an efficient route for prion neuroinvasion. Following intralingual inoculation of the HY TME agent, prion infection was detected in the brain stem one to two weeks after infection. This finding indicated a retrograde spread of the HY TME agent via the hypoglossal nerve to the brain stem. The DY TME agent, which does not replicate in tissues of the lymphoreticular system (LRS), also spread to the brain stem from the tongue suggesting a direct infection of the brain via cranial nerves independent of LRS infection (Bartz, Kincaid et al. 2003; Mulcahy, Bartz et al. 2004; Dejoia, Moreaux et al. 2006).

## **Primates**

### ***CI Lasmézas***

In macaques experimentally infected with BSE and vCJD agents, PrP<sup>TSE</sup> was detected in tissues of the LRS at levels 10 to 1000 times lower than those in the brain. PrP<sup>TSE</sup> was also detected in the enteric nervous system of the digestive tract, in adrenal gland and in muscle tissue including that of the heart and tongue of these animals as well as in those primates infected with agents of sporadic or iatrogenic CJD. This distribution of PrP<sup>TSE</sup> was common to all strains of human TSEs tested but at very low levels ( $\leq 1/10,000$  fold lower than the concentration in brain). Thus, in the macaque model, only the vCJD agent appears to be significantly lymphotropic. The small amounts of PrP<sup>TSE</sup> deposited in different organs of monkeys infected with all human prion strains examined were consistently associated with the peripheral nervous system and might be due to secondary nervous spread of the agent (Herzog, Riviere et al. 2005).

## **Human tissue bioassay**

### ***JW Ironside***

PrP<sup>TSE</sup> has been detected in skeletal muscles of humans with sporadic CJD (sCJD, eight of 32 cases (Glatzel, Abela et al. 2003)), iatrogenic CJD (iCJD, one of five cases) and vCJD (eight of 17 cases) but not in cardiac muscle (Peden, Ritchie et al. 2006). In samples from cases of sCJD and vCJD there was a possible direct relationship between duration of illness and presence of PrP<sup>TSE</sup> in muscle although one case of very short duration was also positive. Attempts to determine the anatomic sites of deposition more precisely suggested that at least some PrP<sup>TSE</sup> was present in intramuscular nerves rather than in muscle fibres themselves. In vCJD, infectivity was detected by bioassay using RIII mice; the highest levels were found in brain ( $10^{5.1}$  IC mouse ID50/g). Lower levels have been detected in spinal cord, trigeminal ganglion, dorsal root ganglion, peripheral nerve, spleen, tonsil, and lymph nodes ( $10^{2.7-3.8}$  IC mouse ID50/g). Negative results were obtained in transmission attempts with appendix, buffy coat, plasma, CSF and bone marrow, although those efforts did not exclude the possibility that small amounts of infectivity, below the limit of detection by the assay in RIII mice, might have been present (Bruce, McConnell et al. 2001; Bruce and Ironside, unpublished).

## **TSE infectivity in urine**

### ***PL Gambetti***

Detection of PrP<sup>TSE</sup> (using the 3F4 mAb) was reported in 2001 in urine of patients having familial CJD with the PRNP E200K mutation and in animal models of scrapie (Shaked, Shaked et al. 2001). Those results were soon challenged and attributed to an artifactual binding of labeled secondary antibody either to human IgG light chain (Serban, Legname et al. 2004) or to bacterial proteins in the urine (Furukawa, Doh-ura et al. 2004). However normal PrP is present in urine at a concentration about 10 ng/ml, mainly found as a diglycosylated truncated form lacking the N-terminal region in which the 3F4-reactive epitope is located (Gambetti, unpublished); that finding casts further doubt on the reports of PrP<sup>TSE</sup> in urine.

## **TSE Blood Infectivity**

### ***Transfusion transmission of vCJD***

#### ***RG Will***

As of May 1, 2006, the total number of vCJD cases in the UK was 161, of which 147 patients had been eligible to donate blood (i.e., aged  $\geq 17$  years). Thirty-one were reported by relatives to have been blood donors and 23 were traced in donor records (including three donors not identified by relatives). The number of recipients of labile blood components from donations of 18 identified vCJD cases where both recipient and component information is available was 66 with a median recipient age of 68 years (age range 17-99); 14 recipients were under 50 years old. Thirty-six recipients survived more than two years and 20 lived more than five years after receiving the transfusion. These figures change on a regular basis as some recipients live longer.

Among 18 recipients surviving more than five years after transfusion, three presumptive vCJD transmissions have now been reported, indicating a very high attack rate. The first case, identified in December 2003, developed clinical symptoms 6.5 year after transfusion with non-leukodepleted red cells from a healthy donor who became ill with vCJD 3.5 years after the donation (Llewelyn, Hewitt et al. 2004). The second case died of an intercurrent illness in 2004 without any neurological clinical symptoms but with positive immunohistochemistry tests for PrP<sup>TSE</sup> in spleen and a cervical lymph node, suggesting the presence of a sub-clinical or pre-clinical infection five years after a transfusion with non-leukodepleted red cells from another healthy donor who developed vCJD 18 months after the donation (Peden, Head et al. 2004). The second transfusion-transmitted case was heterozygous for methionine and valine at PRNP codon 129, in contrast to all clinical cases of vCJD tested to date, in which the PRNP-129 genotype has uniformly been methionine-homozygous.

Since the WHO Consultation, a third presumptive transfusion-associated case of vCJD has been identified in the UK, in a patient who developed vCJD some eight years after receiving a transfusion of non-leukodepleted red blood cells (RBC) from a third healthy donor who developed symptoms of vCJD around 20 months after donating the blood (Health Protection Agency UK, 2006).

To summarize, there have been three transfusion-associated vCJD infections recognized in the UK involving recipients of non-leukodepleted RBC from three different donors incubating vCJD; the blood was donated 1.5 to 3.5 years before signs of illness first appeared in the donors, and vCJD appeared in two recipients more than 6.5 years after the transfusions (with evidence of a pre-clinical or sub-clinical vCJD infection in the third recipient).

Since 1996 UK authorities have taken several precautionary measures to maintain the safety of transfusion and blood products: (1) instituted withdrawal and retrieval of any blood components, plasma derivatives or tissue-derived products remaining from any donor who later developed vCJD (December 1997); (2) imported plasma from the USA for fractionation to manufacture derivatives (announced May 1998, implemented October 1999); (3) introduced leukodepletion of all blood components (announced July 1998, implemented Autumn 1999); (4) imported all fresh frozen plasma (FFP) from the USA to treat patients born on or after January 1, 1996 (announced August 2002, introduced in Spring 2004); (5) promoted the appropriate use of blood and tissues and alternatives throughout the UK National Health Service; (6) deferred transfusion recipients as blood donors (2004); and (7) advised some recipients of plasma products not to donate blood or organs (2004). Step 7 was precautionary, because no cases of vCJD have been recognized in persons who used plasma derivatives in the UK or other countries.

Blood donors who later became ill with vCJD have also been identified in other countries: three in France, one in Ireland, one in Spain and one in Saudi Arabia. No transfusion-transmitted cases have been attributed to blood components from those donors. However, the implications of transfusion-transmitted vCJD for other countries should be assessed.

### **Scrapie or BSE-affected sheep**

#### **S McCutcheon**

Infectivity has been studied with blood of sheep naturally infected with scrapie (sheep having the scrapie-susceptible prion-protein-encoding (Prnp) genotype VRQ/VRQ). Those sheep had 100% prevalence of scrapie with a mean incubation period of  $1291 \pm 166$  days. Other studies used blood of sheep with the Prnp genotype ARQ/ARQ, each infected perorally with a 5-g dose of BSE-infected cattle brain; those animals have had a 60% rate of clinical BSE after incubation periods ranging from 550-650 days. TSE-free New Zealand sheep have been used as blood recipients (Hunter, Foster et al. 2002).

To update results of the transmission studies, five of 24 recipients of blood from BSE-infected sheep have shown clinical signs of TSE infection (incubation periods ranging from 531-610 days) and had positive confirmatory pathology, while there have been nine confirmed cases of transmission among the 21 recipients of blood from scrapie-infected sheep (incubation periods ranging from 575-1138 days). The provisional estimated transmission rate is 43% for scrapie

and 20% for BSE (however, if results from pre-clinical blood donors are excluded, the rate of transmission for BSE reaches 60%). So far, no negative-control sheep have shown evidence of TSE. The surviving recipient sheep have now lived for 1151-2326 days post-transfusion. Extended incubation periods might be expected in animals that received low infectious doses. These studies also indicate that sheep infected by the IV route have deposition of PrP<sup>TSE</sup> in peripheral tissues much more limited than that seen following oral BSE infection or in sheep with natural scrapie. In animals transfused with blood from BSE-infected donors, weak or negative PrP<sup>TSE</sup> signal has been observed in tonsils and mesenteric lymph nodes. Data obtained with sheep confirm observations of humans and indicate that transfused blood poses a risk of TSE and suggest that sheep may provide a useful model to investigate blood-borne TSEs. The effect of leukodepletion on transfusion transmission of scrapie and BSE from sheep to sheep is under ongoing study.

### **Experimental models of TSE diseases**

#### **MT Bishop**

To assess the risk of human-to-human transmission of CJD (iatrogenic CJD) in an experimental model, new lines of human PrP Tg mice were developed, each expressing a single copy of the human PRNP gene with genotypes MM, MV or VV at codon 129 and under control of normal mouse Prnp gene regulators. As predicted, mice of all three codon-129 genotypes were susceptible to infection with sporadic CJD (sCJD) agent. Tissues from sCJD MM1 cases ((Parchi-Gambetti classification (Parchi, Giese et al. 1999), comprising ~70% of all CJD cases)) transmitted disease efficiently to mice of all three genotypes. Tissue from MV2A or VV2A sCJD cases also transmitted disease to Tg mice of all genotypes, after short incubation times in some VV mice. Inoculations of tissue from a human vCJD case also transmitted infection to each of the same three lines of Tg mice, however only a very few mice of MM and MV genotypes—none of the VV mice—became clinically ill after long incubation times. Since most MM and MV mice and one VV mouse inoculated with vCJD tissue eventually demonstrated PrP<sup>TSE</sup> in brain by immunohistochemistry, all Tg mouse genotypes must be considered susceptible to vCJD infection. It is interesting to note that Tg mice of all three lines resisted infection with BSE agent, while similar Tg mice expressing the bovine PrP gene sequence were highly susceptible to BSE.

The very long asymptomatic survival of most vCJD-infected MV and VV mice suggests that infected humans with those genotypes might also have a protracted sub-clinical course. The histopathological characteristics of vCJD in MV and VV mice differed substantially from those of MM mice. MM mice infected with vCJD had more prominent vacuolation with florid plaques not seen in the other genotypes; the same difference might be expected for human vCJD cases with MV and VV genotypes. However extracts of brain tissues from vCJD-infected mice of all three genotypes studied by immunoblotting had the same type-2B PrP<sup>TSE</sup> found in PRNP codon-129 MM humans with vCJD. Tg mice infected with vCJD agent might also prove useful as a model for future studies of blood-borne vCJD (Bishop, Hart et al. 2006).

#### **RG Rohwer**

In the hamster scrapie model (hamster-adapted scrapie strain 263K (Kimberlin and Walker, 1977)) infectivity in blood has been estimated to be about 10 infectious doses (ID) per ml. Repeated direct measurements of infectivity in large volumes consistently found between two and 27 intracerebral infectious doses/ml of whole blood with an average of 10 ID/ml—the infectivity units were Poisson-corrected individual doses and not ID50, because large volumes were divided and assayed completely by intracerebral inoculation into many hamsters. For a human blood unit of 500 ml, 10 ID50/ml corresponds to 3 – 4 log<sub>10</sub> ID/unit. About 30% of infectivity was detected in plasma and 25 % in red cells (RBC) contaminated by white blood cells (WBC) and plasma. Additional studies suggest that there is probably no intrinsic scrapie infectivity in either RBC or platelets of hamsters and infectivity appeared to be distributed equally between plasma and WBC. However, infectivity associated with WBC was easily washed away (Gregori and McCombie, 2004). Thus the real distribution of intrinsic infectivity in blood of scrapie-infected might be 96% in plasma and only 4% in WBC. The infectious titre in blood appeared to increase linearly, first detected at

about 2 ID/ml of whole blood during the middle of preclinical stage of the disease and rising to 6 ID/ml during clinical illness. Transfusions of blood were 50-fold to 100-fold less efficient than direct IC inoculation for infecting hamsters with scrapie. Note that 2 ml transfused into a hamster represented some 33% of its total blood volume, which suggests that transfusion transmission rate for scrapie-infected hamster blood must be low (three successful scrapie transmissions of 100 total 2-ml transfusions); in contrast, transmissions with scrapie-infected and BSE-infected sheep blood transfused 450 ml volumes, representing only 5-6% of total blood volume of the sheep, but they transmitted far more frequently.

## 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<sup>TSE</sup> in blood. The first method relied on a misfolded protein diagnostic (MPD) assay using a conformationally responsive palindromic polypeptide ligand able to detect PrP<sup>TSE</sup> 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<sup>TSE</sup> in plasma was estimated at 1 ID/ml.

The second method is a research prototype that detects proteinase K-resistant PrP<sup>TSE</sup> in blood. The first step concentrates soluble oligomers of PrP<sup>TSE</sup> 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<sup>TSE</sup> 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<sup>TSE</sup> specific binding reagents to bind PrP<sup>TSE</sup> in plasma without proteinase K treatment. After dissociation from the beads, PrP<sup>TSE</sup> 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<sup>TSE</sup> at least 6600-fold. One round of PMCA was reported to increase sensitivity of detection 2500 fold when compared to titre by 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<sup>TSE</sup> 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<sup>TSE</sup> 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<sup>TSE</sup> capture, wash, elution, denaturation and final detection by ELISA. Spiked samples containing 1-10 mg vCJD spleen/ml of plasma (equivalent to 10<sup>2</sup>-10<sup>3</sup> 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<sup>TSE</sup> (Korth, Stierli et al. 1997; Nazor, Kuhn et al. 2005) to capture and enrich it from serum or plasma (200- $\mu$ l aliquots) and subsequent detection with a labelled secondary anti-PrP specific antibody. The assay uses either ELISA or fluorescence-activated cell sorting formats. PrP<sup>TSE</sup> 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.

## 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 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 unblinded 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 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

too limited to serve as international reference materials, which would require special prospective collection supported by commercial or government funding not currently available.

### ***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 PrP<sup>TSE</sup>, 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 PrP<sup>TSE</sup> 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.

## **Evaluation of TSE removal procedures**

### ***Labile blood products***

#### ***M Turner***

Blood services attempting to reduce the risk of person-to-person transmission of vCJD by blood components must compare predicted effects of risk reduction strategies with the competing risk of contributing to product shortages and the direct and indirect effects of increased costs. Measures taken in the UK so far include the withdrawal from the market and retrieval of labile components from donors later diagnosed with vCJD, deferral of donors who spent more than some specified amount of time in countries deemed to be at high risk for BSE, universal depletion of leukocytes from blood components, importing Fresh Frozen Plasma (FFP) for treating either certain groups of recipients—those born after elimination of most food-borne BSE risk from the country—or all recipients under consideration, and deferral of donors transfused after the presumed start of the BSE outbreak in 1980. Possible additional measures that might be taken include importation of all FFP from BSE/vCJD-low risk countries, using only apheresis platelets, and modifying the standard preparation of red blood cells (RBC) concentrates to remove as much plasma as possible.

At least 2 commercial enterprises have reported development of filters that might remove infectivity from RBC after preliminary leukodepletion; the performance of those filters has not yet been fully validated and clinical trials have not been reported. On theoretical grounds, removal of at least 3 log<sub>10</sub> of infectivity from RBC concentrates is likely to reduce significantly the risk of blood-borne person-to-person transmission of vCJD. UK authorities have proposed the following specifications (among others) for “prion-reduction” filters: (1) removal of ≥3 log<sub>10</sub> of infectivity from blood spiked with brain-derived material from animals with TSEs; (2) removal of ≥1 log<sub>10</sub> of infectivity, and/or removal of PrP<sup>TSE</sup> detected by immunoblotting or other test if a blood-based assay has been developed and validated, from blood of animals with an endogenous TSE infection; (3) development of a surrogate marker to monitor effectiveness of infectivity reduction (encouraged); (4) maintenance of acceptable RBC quality (essential), as evidenced by normal survival of RBC in volunteers as measured with a radio-chromium-recovery test, and other standard assays for

RBC membrane changes including tests at 42-day expiry, and eventually clinical trial comparing filtered with untreated RBC for multiply transfused recipients.

### **Plasma derivatives**

#### **B Flan**

To date there has not been a single reported case of transmission of a TSE, including vCJD, from the use of a plasma-derived medicinal product, and the risk of such transmission is considered to be extremely low. During manufacture, plasma products undergo a number of purification steps with a capacity to remove pathogenic agents that might be present in the starting material. Despite the fact that the physicochemical nature of the prion in blood is not yet elucidated, the known physicochemical properties of brain-derived PrP<sup>TSE</sup> (hydrophobicity, insolubility, adsorption to various matrices and aggregation) allow a prediction of the possible behavior of infectivity during the fractionation and purification of plasma proteins. There is now a large body of convergent scientific data to document the highly efficient prion removal capacity of classical plasma fractionation methods; these data were presented and discussed in an EMEA TSE expert meeting (EMEA, 2004a) and have been reviewed (Foster, 2004; Flan and Aubin, 2005). Published experimental studies were performed by different teams worldwide, using slightly different manufacturing processes and different experimental models; the fact that their results were so similar gives confidence in the interpretation of the data. The relevance of experiments using brain-derived spiking materials for inferring the probable behavior of TSE agent in blood, and the influence of different spike preparations, TSE agent strains and animal models on results have all been considered in the scientific literature, providing a scientific basis for the European Guideline on the investigation of manufacturing processes for plasma-derived medicinal products with regard to vCJD risk (EMEA, 2004b).

Methods used to detect the TSE agent in these experiments were either immunochemical detection of pathogenic prion protein, recently improved (Hartwell, Nelson et al. 2005), or bioassays for infectivity. Several laboratories showed that results obtained using immunoassays for PrP<sup>TSE</sup> correlated well with those from infectivity studies in animals. Efforts are in progress to develop *in vitro* cell culture infectivity assays that might eventually offer a suitable alternative to time consuming *in vivo* bioassays (Klöhn, Stoltze et al. 2003; Flan, unpublished).

The efficient capacity of precipitation steps to remove prions, first shown by studies of partitioning of endogenous infectivity using a rodent model, has been well documented for ethanol fractionation in the manufacture of albumin and immunoglobulins and for other precipitation steps like caprylic acid (Trejo, Hotta et al. 2003) or polyethylene glycol (PEG) treatments. It has been shown that filter aids used to remove precipitates reduce TSE infectivity as well (Gregori, Maring et al. 2004); depth filtration also removed large amounts of prions in a range of applications. Care must be taken in extrapolating these data, however, as no efficacy was shown under certain other conditions (Van Holten, Autenrieth et al. 2002).

A number of studies also showed the efficacy of several chromatography steps, mainly ion-exchange chromatography (IEC) (Foster, Griffin et al. 2004). Nanofiltration also significantly reduced TSE infectivity or PrP<sup>TSE</sup> in various applications with different experimental models (Gregori, Maring et al. 2004; Van Holten and Autenrieth 2003). While addition of harsh dispersing detergents and ultrasonication to experimental procedures dramatically reduced the efficacy of nanofiltration, those treatments are not used in the manufacture of plasma products, and prion removal capacity remained after solvent/detergent (SD) treatment of spiking materials.

A cumulative effect on prion removal was documented for a combination of two depth filters in the manufacture of an IgG preparation (Gregori, Maring et al. 2004), but that effect was not observed in another experiment in which filtration was introduced at a different stage of the process and under different conditions (Reichl, Foster et al. 2002). In a combined experiment, we showed that neither SD treatment nor IEC negatively affected the efficacy of a nanofiltration step, and the data suggested a cumulative effect of chromatography and nanofiltration (Porte, Aubin et al. 2005). A cumulative effect of precipitation steps in Cohn fractionation of plasma has also been reported (Cai, Miller et al. 2002).

Regarding newer TSE removal procedures, while modest inactivation of TSE agent was reported after gamma irradiation (Miekka, Forng et al. 2003), a more promising approach, using affinity ligands, yielded more encouraging results recently. Data from other recent studies on TSE removal further confirmed the probable efficacy of various manufacturing processes commonly used in plasma fractionation to remove TSE infectivity. Additional techniques are currently under development.

Uncertainties about the nature of the agent in blood must be kept in mind when interpreting data from experiments spiking brain-derived material, and additional studies are needed to clarify this issue. When extrapolating from published experimental data, caution regarding relevance to actual manufacturing processes must always be observed. However, the consistency of data currently available and increasing knowledge regarding the mechanisms of action for specific steps used to fractionate plasma suggest that processes used to manufacture plasma derivatives have a substantial capacity to remove prions.

### **Decontamination: new procedures**

#### ***JP Deslys***

The unique resistance of the vCJD agent to usual decontamination techniques poses a very difficult infection control challenge not only to healthcare facilities but also to the pharmaceutical industry and, more widely, to all industries using human-derived and ruminant-derived materials. The drastic procedures usually recommended to decontaminate materials potentially exposed to TSE agents (1M sodium hydroxide or 2% sodium hypochlorite for 1 hour with 134°C autoclaving for at least 18 minutes) are not applicable to treatment of most fragile devices and in many other situations. Hence, new procedures must be developed to fill this gap.

It would be helpful to reach a consensus on validation standards—data acceptable to healthcare and national regulatory authorities—for practical methods to decontaminate TSE agents. Different experimental models have been employed to compare the efficiency of classical prion decontamination methods and newer procedures that do not damage delicate devices. Reproducible results have been obtained with a 263K hamster model and a BSE mouse model using IC implantation of wires contaminated with infected brain, dried, and then exposed to different decontamination treatments. We observed that treatments with limited concentrations of alkali or stabilized bleach, when properly formulated with detergents and used at specified temperatures, exhibited similar efficiency for removing prions as did classical NaOH or NaOCl treatments (Fichet, Comoy et al. 2004). Because the new methods required shorter exposure times and were compatible with delicate materials, they appear to be potentially suitable for practical use. A new dry system based on vaporized hydrogen peroxide under vacuum appeared to be more efficient for inactivating TSE agents than conventional steam autoclaving and preserved the function of heat-sensitive electronic devices. These new experimental models, combined with *in vitro* protocols that detect PrP<sup>TSE</sup>, allow comparisons effectiveness of new versus standard TSE decontamination regimens and exploration of the basic mechanisms of action involved. Unresolved issues include the influence of various cleaning methods, product formulations and sequence of treatments on final effectiveness of decontamination. Appropriate waste disposal after TSE decontamination is also an important issue.

Thus, new procedures for prion decontamination might offer improved efficiency. They must be compared with conventional decontamination procedures using standardized experimental validation systems. After new procedures are validated they should be considered for recommendation by national authorities.

### **Assessment of the risk of transmission of vCJD via blood, blood components or plasma-derived products**

#### ***A Farrugia, Australia***

All assessments of the risk of BSE in the Australian ecosystem and food chain have agreed that it is among the lowest in the world. Nevertheless, the globalization of regulatory decision making, together with Australia's multi-ethnic society, required regulatory intervention to ensure that the

blood supply remains safe from vCJD. Assessment of the risk-benefit balance of various donor deferral options revealed that maintaining a sustainable blood supply was possible only with a minimalist donor deferral policy based on residence in the UK during 1980 to 1996. Following implementation of this policy, risk assessments for the various plasma derivatives revealed that the highest exposures to vCJD were expected for patients receiving Factor VIII concentrate. This conclusion contributed to a policy decision, implemented in 2004, to convert to recombinant FVIII. Assessments comparing the risk reductions obtained by leukocyte depletion of blood and further donor deferrals continue to influence decisions. In the absence of firm data on blood infectivity and the current lack of a vCJD screening test, risk assessments must draw on a range of inputs regarding the factors leading to infection and disease. The risk assessments assist in the development of measures that integrate and complete other aspects of decision making in blood policy (Farrugia, Ironside and Giangrande, 2006).

### ***S El Saadany, Canada***

The Public Health Agency of Canada convened a third session of the Statistics and Risk Assessment Section's International Expert Advisory Group on Risk Modeling meeting in Ottawa, Canada on February 24-25, 2005. Experts from Australia, Austria, Canada, France, the Netherlands, UK and USA attended. This meeting examined the various vCJD risk estimates and mathematical risk modeling techniques for vCJD used by France, UK, USA and Canada. A working group was established to identify parameters common to more than one model and potential missing parameters. The group also looked at variation in the assumptions and the mathematical structures of the models. To test the various models, the working group ran a number of different data sets estimating risk for several clotting factors including FVIII and FXI. Differences in the models were noted regarding assumptions both about infectivity in blood and vCJD prevalence in plasma donors. A report created by the working group delineated the outcomes of the meeting and includes Canada's own risk assessment. One issue that emerged was the special importance of risk communication. There is a great need to develop risk communication statements for regulators and physicians communicating to the public their likelihood of being infected by exposure to blood products and contracting the vCJD.

### ***JH Trouvin, France***

Analysis of the risk of transmission of the CJD agent by blood and blood products—both labile transfusion products and plasma-derived medicinal products (PDMP)—was first undertaken in France in 1997 and has been regularly updated since then. The first public report was issued in December 2000 (<http://afssaps.sante.fr/ang/pdf/mcj05.pdf>) and the analysis has since been revisited on several occasions. The latest updates, in February and November 2004 ([http://afssaps.sante.fr/ang/pdf/vmcjsg\\_05.pdf](http://afssaps.sante.fr/ang/pdf/vmcjsg_05.pdf)), were triggered by the first two reports of probable transfusion-transmitted vCJD in the UK and the notification of two French cases of vCJD in persons who had been blood donors on several occasions before clinical onset. Even though the likelihood of transfusion-transmitted vCJD had been anticipated in the very first French risk analysis, a group of independent experts was asked to re-evaluate the risk of transmission of vCJD by blood products, in order to propose any new measures deemed necessary to decrease the risk further. The new risk analysis was based on the following parameters: (i) the estimate of vCJD cases expected in the French population, (ii) the possible CJD infectivity titres in blood, (iii) the probable distribution of infectivity in blood and its components, (iv) the precautionary measures already in place in France, (v) for PDMP, the probable infectivity reduction factors provided by those steps in the plasma fractionation process shown to inactivate or remove the infectious agent, and (vi) the route of administration and the annual dose received by a patient treated with a PDMP.

As regards the quantitative assumptions used for the risk analysis, it is noteworthy that the figures used in 2000 have been modified as follows: the number of clinical vCJD cases expected to occur in France has been lowered (Chadeau-Hyam and Alperovitch, 2005); however, the worst-case scenario of 300 cases during the next 60 years has been maintained. The probable level of infectivity in whole blood has been lowered to 20 to 30 infectious units per ml (Cervenakova, 2003). For the distribution of infectivity in blood components, the original hypothesis that 90%

of infectivity is concentrated in the buffy coat has been modified and a more reasonable hypothesis of 30% in buffy coat and 50% in plasma (Gregori, McCombie et al. 2004) adopted. In addition, the leukoreduction of whole blood has been considered to decrease the infectivity in plasma by 50%. Finally, regarding efficiency of the route of administration, whereas the IV route was initially considered to be 10-fold less infective than the IC route, both routes are now considered to be equally efficient in transmitting vCJD (Herzog, Salès et al. 2004).

The conclusion of the latest risk analysis for blood cellular components remains unchanged from earlier assessments: one unit of RBC or platelet concentrate is estimated to contain more than one infectious unit if the donor was incubating vCJD. The residual risk of receiving one infectious blood unit by transfusion depends on the prevalence of persons incubating vCJD in the blood donor population. Assuming that blood donors are a random sample of the French population, that the 300 asymptomatic infected subjects are restricted to the general population aged 18 to 65 years (i.e., the 36 million persons eligible to donate blood), and that blood from an asymptomatic donor is infective throughout the incubation period, it follows that the prevalence of pre-clinical infectious persons among the potential blood donor population would be 300/36 million persons, leading to the estimate that one blood donor per 120,000 might be infectious.

As regards PDMPs prepared by the French fractionator, the risk analysis was updated by assuming a higher infectivity titre in plasma (10 IV infectious units per ml), allowing for a reduction in infectivity by a nanofiltration step used in the manufacturing process of some PDMPs, and evaluating capacity of some other purification steps to remove infectivity. It should be noted that the residual vCJD risk for PDMPs was estimated using a worst-case assumption that every single pool of French plasma used to prepare the products would contain at least one donation from an infected donor; this assumption must be considered as very conservative, because an estimate of the reservoir of infectious donors in France suggested that only one in every five to eight plasma pools would actually be contaminated. Overall, changes in some figures in the working assumptions had no significant consequences for the estimated residual risk. For PDMPs, the estimated residual risk of infectivity in the final product suggested a three-log to five-log margin of safety.

In conclusion, two distinct situations can be considered: (1) transfusion of labile blood components, for which the probable risk of receiving one infectious unit was estimated at 1/120,000 transfusions but for which the benefit outweighs the risk so long as transfusion is restricted to well justified indications and patients are informed both before and after treatment, and (2) PDMPs, for which the risk is considered as minimal.

### ***J Löwer, Germany***

Countries with cases of BSE in cattle have implemented effective measures to prevent transmission of BSE to bovines and other animals. Therefore, in most, if not all, countries the annual numbers of BSE cases are clearly declining. Procedures are also in place to prevent the transmission of BSE to humans by food. The effectiveness of these measures has been demonstrated by the decrease in new vCJD cases recognized in the UK. No case of BSE transmitted to humans has been linked to medicinal products or medical devices, probably due to the strict control of bovine material used in the preparation of these products. Secondary transmissions of vCJD from human to human, except those cases mentioned below, have not been reported either. The control of human materials used to prepare medicinal products and medical devices and improved methods used to sterilize invasive instruments may have helped prevent transmission of vCJD.

In a number of animal model systems, TSE infectivity has been shown in blood. In addition, three human cases, described in the UK, were most probably infected with the vCJD agent by transfusion of red blood cell concentrates. The donors, who developed vCJD 18 months to three years after donation, were most probably infected by food. Due to the long incubation periods of vCJD, it is not unlikely that more people are silently infected with vCJD and might transmit the agent by blood donations. However, if the measures described above are as effective as expected, no new secondary vCJD transmissions should occur except by blood transfusion. A simple way to interrupt the transmission of vCJD by blood would be to screen donors with a sensitive diagnostic test. However, such tests will not become available for at least several years.

When transmission of vCJD by blood transfusion cannot be avoided due to the lack of adequate diagnostic tools, two questions arise: (1) Is it possible that vCJD might become an endemic disease propagated only by blood for transfusion? (2) Would the prevention of tertiary and higher-order transmissions of vCJD (transmission by blood from donors previously infected with vCJD by a blood transfusion) effectively reduce transfusion-transmitted vCJD (from donors infected by food or by blood)?

To address these questions, Prof. Klaus Dietz (University of Tübingen, unpublished) developed a mathematical model using established risk assessment procedures. The demographic data used for Germany assumed a vCJD epidemic similar to that in France (as there have been no vCJD cases recognized in Germany to date preventing a direct estimate of characteristics of a vCJD epidemic). A number of basic parameters were calculated, e.g., the age-specific death rate, the age-specific rate of first becoming a donor and of terminating donations, the age dependence of transfusion risk, and the transfusion-associated mortality. A limited study was conducted to determine whether individuals with a transfusion history have a higher probability of subsequently becoming donors themselves. This widely believed relationship could not be substantiated. The model yielded the following findings regarding the above-mentioned questions:

1. Under realistic conditions for Germany, vCJD would not become an endemic disease propagated only by blood for transfusion.
2. The exclusion of donors with a transfusion history would only marginally reduce the number of donors infected with vCJD by blood transfusion.

The latter statement must be considered carefully. In Germany, where there have been no cases of vCJD and where the epidemic assumed in the risk model is a worst case, it might be justified not to exclude donors with a history of transfusion. However, the conditions assumed for Germany might not apply to other countries, so each country should evaluate its own situation in order to reach appropriate risk management decisions.

### ***P Bennett, UK***

From the first identification of vCJD as a new disease, estimates of its potential impact on public health assumed that it might be transmissible from person to person. The prudence of this precautionary view was unfortunately borne out by subsequent events, at least as regards the transmissibility of vCJD by donated blood. Measures to mitigate the risk of secondary (person-to-person) transmission of vCJD have been informed by a series of risk models developed—with external collaboration—within the UK Department of Health. These models consider the potential transmission risks associated with hospital surgery, transfusion of blood and blood products, use of donated bone and other tissues or organs, and “high street” (outpatient) dentistry. All these models addressed a wide range of scenarios, reflecting the continuing scientific uncertainties around both the prevalence of infection, the pathogenesis of vCJD and the biology of its causative agent. Great uncertainty remains regarding infectivity in blood, interactions between different transmission routes and implications of a possible prolonged carrier state (sub-clinical or pre-clinical infection) for vCJD.

### ***D Scott, USA***

Although vCJD has not been reported in recipients of plasma derivatives, TSEs have long incubation periods, especially after low-dose exposures. If plasma derivatives pose a risk of exposing recipients to vCJD agent, the risk is expected to be greatest for products that (1) have low potential for TSE infectivity clearance by manufacturing, (2) are administered in large amounts for treating chronic conditions, and (3) are most likely to have been manufactured from pools of plasma containing a donation from an individual incubating vCJD. FDA developed and publicly presented risk assessment model for vCJD transmission by plasma derivatives (accessible at <http://www.fda.gov/ohrms/dockets/ac/cber05.html#TransmissibleSpongiform>). Three main categories of information are needed to assess vCJD risk: (1) an estimate of the amount of infectivity in starting material, (2) the amount of infectivity cleared during manufacturing, and (3) the amount of product administered to a recipient. Within each category there is substantial uncertainty, which has been

represented as a distribution or range of possible values. Where data were not available, simplifying assumptions were used. Monte Carlo analysis of the model was conducted. For a factor XI (FXI) risk assessment, simulations of 10,000 iterations were run. The model provided predictions of estimated exposure to vCJD agent in the form of IV ID50 in patients treated with a UK-manufactured plasma-derived FXI. Because an accurate dose-response relationship (or hazard characterization) for vCJD exposure and the probability of human illness is not known it was not possible to predict with any confidence the probability of vCJD infection and illness in exposed individuals.

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