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Protocol for further laboratory investigations into the distribution of infectivity of Atypical BSE[1]

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ABSTRACT

Information on the pathogenesis and tissue distribution of Atypical Bovine Spongiform Encephalopathy (BSE) in cattle through the study of field cases and experimental transmission studies is lacking. The latter are limited to transmission of Atypical BSE through intracerebral (i.c.) inoculation of cattle. All data currently available relate to the presence or absence of PrP^{Sc}, but do not quantify relative amounts of PrP^{Sc} or levels of infectivity. A laboratory protocol for further studies is recommended, to allow the assessment of the relative infectious titre, PrP^{Sc} accumulation and prion seeding activity in the tissues of cattle that developed H-BSE or L-BSE (using posterior brainstem as a reference). Tissues to be covered by those studies are categorised in three priorities, based on their inclusion in the list of specific risk material in cattle, on the presence of infectivity, or PrP^{Sc} presence, demonstrated in Atypical BSEs or other Transmissible Spongiform Encephalopathies (TSEs) in ruminants, and on the importance in terms of input into the food chain in the EU. The protocol provides details in terms of the minimum number of animals to be tested, processing and preparation of tissues, and methods to be used to identify abnormal PrP and quantify infectivity, also depending on the expected level of infectivity and amount of tissue available for analysis. It is recommended that, through the implementation of the protocol, information should also be obtained on the performance of currently validated rapid tests for TSE active surveillance in cattle/bioassay for detecting H-BSE and L-BSE agents.

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KEY WORDS

Atypical BSE, cattle, H-BSE, L-BSE, laboratory protocol, prion

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SUMMARY

Following a request from the European Commission, EFSA was asked to provide scientific and technical assistance on a protocol for further laboratory investigations into the distribution of infectivity of Atypical Bovine Spongiform Encephalopathy (BSE).

The European Union Reference Laboratory (EURL) for Transmissible Spongifom Encephalopathies (TSEs) conducted experiments (referred to as the EURL study) that resulted in the collection and storage of reference material originating from cattle experimentally infected with Atypical BSE (H-BSE and L-BSE). With the intention to generate relevant data that could inform further policy options regarding BSE, in particular as regards rules on specified risk material (SRM), DG SANCO wished to explore the possibility to submit these tissue samples to further investigations. EFSA was therefore asked to propose a laboratory protocol to perform new studies aimed at investigating the presence, distribution and relative level of infectivity of Atypical BSE (H-BSE and L-BSE). EFSA was also expected to reflect on whether the tissues available from the EURL study are sufficient for the purpose and/or suggest that investigations on other tissues might be possibly needed.

Data relating to the prevalence and geographical distribution of Atypical BSE in the European Union (EU) are incomplete and subject to variation owing to the ongoing retrospective typing of BSE cases. So far, 80 cases of Atypical BSE have been reported by EU Member States from 2001 to 2014. All Atypical BSE cases have been detected by active surveillance, typically in animals over eight years of age, with a similar number of cases detected each year.

This report provides an overview of the biological material collected from field cases of Atypical BSE and transmission studies, both in the framework of the EURL study and additional published scientific studies. It was concluded that information on the pathogenesis and tissue distribution of Atypical BSE in cattle through the study of field cases and experimental transmission studies is lacking. The latter are limited to transmission of Atypical BSE through intracerebral (i.c.) inoculation of cattle. Where data exist from both field cases and experimental animals (i.e. for L-BSE only), there is good agreement of the data with regard to abnormal PrP distribution. There are no data for field case H-BSE. All data currently available relate to the presence or absence of PrP^{Sc}, but do not quantify relative amounts of PrP^{Sc} or levels of infectivity.

Approaches to quantify Atypical BSE prions in cattle tissues are described and reviewed, including bioassay and *in vitro* methods.

A laboratory protocol for further studies is recommended. Its application would provide elements allowing the assessment of the relative infectious titre, PrP^{Sc} accumulation and prion seeding activity in the tissues of cattle that developed H-BSE or L-BSE (using posterior brainstem as a reference). Tissues to be covered by those studies were categorised in three priorities, based on their inclusion in the cattle SRM list, on the presence of infectivity, or PrP^{Sc} presence, demonstrated in Atypical BSEs or other TSEs in ruminants, and on the importance in terms of input into the food chain in the EU. The protocol provides details in terms of the minimum number of animals to be tested, processing and preparation of tissues, and methods to be used to identify abnormal PrP and quantify infectivity, also depending on the expected level of infectivity and amount of tissue available for analysis.

Applying the protocol only to the tissues obtained through the EURL study would provide information on some but not all the tissues from the cattle SRM list. It would also provide information on some additional tissues not included in the cattle SRM list, but relevant for the food chain. Material from other studies could be used to augment the range of SRM and non-SRM tissues available. It is acknowledged that there is no identified source able to provide all the samples necessary to assess infectivity in tissues belonging to the full cattle SRM list in H- and L-BSE-infected animals, and therefore, to complete this objective, new inoculation experiments with H- and L-BSE agents in cattle would have to be considered. Recommendations on general principles for such new experiments are provided.

In accordance with former EFSA recommendations, it is recommended that, through the implementation of the protocol, information should also be obtained on the performance of currently validated rapid tests for TSE active surveillance in cattle/bioassay for detecting H-BSE and L-BSE agents.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

While the annual number of Classical BSE cases in the EU continues to decrease, more knowledge regarding Atypical BSE (H and L types) appears desirable in order to envisage further policy options regarding BSE control.

In 2009 and 2011, at the request of the European Commission's Directorate General for Health and Consumer Protection (DG SANCO), the European Union Reference Laboratory (EURL) for TSE (AHVLA in the United Kingdom) was tasked to carry out experiments to produce reference material for the EURL and for National Reference Laboratories. In total 12 cattle were inoculated, of which 4 with BSE H-type and 4 with BSE L-type in 2009, and 2 with BSE H-type and 2 with BSE L-type in 2011. Various tissue samples of these infected cattle were collected by the EURL and stored for future use.

Some of these tissue samples have already been tested by the EURL, with at least one laboratory method, as shown in the table in Annex 1[4] More detailed information regarding protocols and experimental results is included in Annex 2[4].

With the intention to generate relevant data that could inform further policy options regarding BSE, DG SANCO wishes to explore the possibility to submit these tissue samples to further investigations. The purpose of the new experiments would be to generate additional data on the presence, distribution and relative level of infectivity of Atypical BSE (H and L types) in the infected cattle from the EURL study.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is therefore asked, in accordance with Article 31 of (EU) Regulation 178/2002, for scientific and technical assistance, i.e. to propose a laboratory protocol to perform new studies aimed at investigating the presence, the distribution and the relative level of infectivity of Atypical BSE (H and L types) in these tissues.

We would like you to deliver this technical assistance by 30 June 2014. EFSA is requested to liaise with the EURL for TSE to facilitate further exchanges of information.

Clarification of the terms of reference

It was clarified that:

- The overall intention of the further investigations would be to generate relevant data that could inform further policy options regarding BSE, in particular as regards SRM rules.

- The EFSA report is expected to reflect on whether the tissues available from the EURL study are sufficient for the purpose and/or suggest that investigations on other tissues might be possibly needed.

[4] Annexes 1 and 2 to the mandate.

ASSESSMENT

1. Introduction

The large-scale testing of nervous tissues from ruminants for the detection of Transmissible Spongiform Encephalopathies (TSEs) has led to the recognition of two molecular signatures distinct from Classical Bovine Spongiform Encephalopathy (BSE) (C-BSE) in cattle. These were termed H-BSE and L-BSE, also known as Bovine Amyloidotic Spongiform Encephalopathy (BASE). Their PrP^{Sc} molecular signature differed from C-BSE in terms of protease-resistant fragment size and glycoform pattern (Biacabe et al., 2004; Casalone et al., 2004; Buschmann et al., 2006). The experimental transmission of these cases to different lines of bovine prion protein (PrP) transgenic (Tg) mice unambiguously demonstrated their infectious nature and most often confirmed their unique but distinctive strain phenotype compared with C-BSE (Beringue et al., 2006; Buschmann et al., 2006; Beringue et al., 2007; Capobianco et al., 2007).

In the following sections, a brief description of the epidemiology, pathogenesis and transmissibility to animal models of Atypical BSE is given. In particular, information provided by EFSA in the past (EFSA BIOHAZ Panel, 2011; EFSA, 2012) is summarised and updated with new evidence that has become available in the last few years.

1.1. Epidemiology

Until recently, typing of cattle BSE cases was not required by European Union (EU) legislation. From July 2013, Regulation (EC) No 999/2001[5] was amended to require that samples from BSE-positive cattle are submitted for TSE classification. Retrospective typing of past BSE cases is also currently ongoing in some EU Member States. Information on BSE type distribution in the EU is therefore incomplete. It originates from data available in the scientific literature (Jacobs et al., 2007; Stack et al., 2009) and data reported by Member States to the European Commission on an ad hoc basis ("EU BSE databases").

Scientific studies report information on the identification of L-BSE in France (Biacabe et al., 2008), Italy (Casalone et al., 2004), Germany (Buschmann et al., 2006), Great Britain (Stack et al., 2013) and Poland (Polak et al., 2008). H-BSE has been reported in France (Biacabe et al., 2008), Great Britain (Terry et al., 2007), Germany (Buschmann et al., 2006), the Netherlands (Jacobs et al., 2007) and Sweden (Gavier-Widen et al., 2008). Outside the EU, Atypical BSE has been also reported in Japan (Yamakawa et al., 2003; Masujin et al., 2008), the USA (Richt et al., 2007), Canada (Dudas et al., 2010) and Switzerland (in a zebu (Seuberlich et al., 2006)).

Data on Atypical BSE cases reported in the EU BSE databases since 2001 are presented in Tables 1 and 2. As indicated above, typing data are incomplete and thus should be considered with caution. In particular, not all EU Member States have provided information on the typing of detected BSE cases, and for other countries the proportion of typed cases is variable. By 2015, these data might be more comprehensive following a request from the European Commission to Member States for re-testing and retrospective classification of all positive bovine isolates in the EU in the years 2003–2009.

[5] Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. OJ L 147, 31.5.2001, p. 1, as last amended.

Table 1: Number of Atypical BSE cases reported by EU Member States in the period 2001–2014 by country and by type (L- and H-BSE) (extracted from EU BSE databases on 1 July 2014). By 2015, these data might be more comprehensive following a request from the European Commission to Member States for re-testing and retrospective classification of all positive bovine isolates in the EU in the years 2003–2009

BSE type	Country	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013[(a)]	2014[(a)]	Total
H-BSE	Austria										1					1
	France[(b)]	1	2	3				1	2	2	2			2		15
	Germany				1										1	2
	Ireland		1								1	2		1		5
	The Netherlands	1														1
	Poland						1			1						2
	Portugal											1				1
	Spain											1	1			2
	Sweden						1									1
	United Kingdom					1		1		1		1		1		5
	Total	**2**	**3**	**3**	**1**	**1**	**2**	**2**	**2**	**4**	**4**	**5**	**1**	**4**	**1**	**35**
L-BSE	Austria							1			1					2
	Denmark				1											1
	France[(b)]		1	1	1	1	2	1	3	2	1		1			14
	Germany		1												1	2
	Italy		1	1				1		1		1				5
	The Netherlands		1	1							1					3
	Poland		1		2	2	1	2				1	2	1		12
	Spain												2			2
	United Kingdom							1			1	1	1			4
	Total	**0**	**5**	**3**	**4**	**3**	**3**	**6**	**3**	**3**	**4**	**3**	**6**	**1**	**1**	**45**
Total Atypical cases (H + L)		**2**	**8**	**6**	**5**	**4**	**5**	**8**	**5**	**7**	**8**	**8**	**7**	**5**	**2**	**80**

(a): Data for 2013-2014 are incomplete and may not include all cases/countries reported.

(b): France has performed extensive retrospective testing to classify BSE cases, which is probably the explanation for the higher number of Atypical BSE cases reported in this country.

Table 2: Number of Atypical BSE cases reported by EU Member States in the period 2001–2014 by type (L-, H- and C-BSE) and target group (extracted from EU BSE databases on 1 July 2014). By 2015, these data might be more comprehensive following a request from the European Commission to Member States for re-testing and retrospective classification of all positive bovine isolates in the EU in the years 2003–2009

BSE type	Target group	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013(a)	2014(a)	Total
H-type	Healthy slaughtered animals	1	1	1	1		1			1	1		1	1	1	10
	Fallen stock	1	2	2		1	1	2	2	2	3	5		3		24
	Emergency slaughter									1						1
L-type	Healthy slaughtered animals		4	1	1	2	2	1		1	1	2	3	1	1	20
	Fallen stock		1	2	2	1	1	5	2	2	3	1	3			23
	Emergency slaughter				1				1							2
C-BSE	Healthy slaughtered animals	282	294	270	175	113	78	39	29	26	13	7	4			1 330
	Fallen stock	400	608	404	312	218	165	91	72	28	23	13	7	2		2 343
	Emergency slaughter	321	512	318	172	125	31	8	6	2						1 495

(a): Data from 2013-2014 are incomplete and may not include all cases/countries reported.

The number of Atypical BSE cases detected in countries that have already identified them seems to be similar from year to year. In France, a retrospective study of all TSE-positive cattle identified through the compulsory EU surveillance between 2001 and 2007 indicated that the prevalence of H-BSE and L-BSE was 0.35 and 0.41 cases per million adult cattle tested, respectively, which increased to 1.9 and 1.7 cases per million, respectively, in tested animals over eight years old (Biacabe et al., 2008). No comprehensive study on the prevalence of Atypical BSE cases has yet been carried out in other EU Member States.

All cases of Atypical BSE reported in the EU BSE databases have been identified by active surveillance testing (59 % in fallen stock, 38 % in healthy slaughtered cattle and 4 % in emergency slaughtered cattle). Cases were reported in animals over eight years of age, with the exception of two cases (one H-BSE and one L-BSE) detected in Spain in 2011/2012. One additional case of H-BSE was detected in Switzerland in 2012 in a cow born in Germany in 2005 (Guldimann et al., 2012).

The generally older age of the identified H-BSE and L-BSE cases, and their apparently low prevalence in the population, suggest that these Atypical BSE forms could be arising spontaneously.

1.2. Pathogenesis

The putative origin of Atypical BSEs (sporadic vs. acquired) influences the extent to which information gathered from experimental studies may represent the real distribution of the agent in the body of naturally affected cattle. Intracerebral (i.c.) challenge would be an appropriate proxy for studying the distribution of the agent if the origin of the disease was spontaneous, and originating in the brain, while oral challenge would be more appropriate if the origin of the disease was through ingestion of infected material.

To date there is no comprehensive information about the pathogenesis of Atypical BSEs in cattle. Some data are available on the peripheral distribution of H- and L-BSE agents in cattle experimentally challenged by the i.c. route, but all of the animals in these studies were allowed to develop clinical disease. Information available from published studies on the transmissibility and distribution of infectivity in tissues of cattle challenged in those experiments are reported in Sections 2.1 and 2.2. No results are currently available for oral transmission experiments of Atypical BSE to cattle. An oral transmission study was initiated by oral challenge of cattle with L-BSE brain homogenate in January 2008 (Panelli et al., 2011), but final results on transmissibility and infectivity distribution are not yet available. The fact that field cases are detected overwhelmingly through active surveillance has also greatly restricted data available on tissue distribution in natural disease, and such data are available only for L-BSE.

The current lack of information on the distribution of infectivity in tissues of Atypical BSE-infected cattle does not allow judgement of whether the current list of bovine specified risk material (SRM), set by EU legislation based on data relating to the pathogenesis and tissues distribution of C-BSE, is fit for the purpose of removing most of the Atypical BSE infectivity from bovine carcasses.

1.3. Transmission studies in animal models other than cattle

Both L-BSE and H-BSE agents are able to propagate in experimentally challenged foreign species. There is a wider host susceptibility for L-BSE, with transmissions demonstrated in mice, sheep, voles, primates and hamsters, as well as in transgenic mice expressing heterologous, i.e. non-bovine, PrP sequences, whereas H-BSE has not yet proven transmissible to sheep, hamsters or humanised transgenic mice.

Proof of principle of the ability of L-BSE to propagate in sheep was provided by the i.c. propagation of an L-BSE isolate in ARQ/ARQ sheep and also in transgenic mice expressing the ovine PrP^C variants. The propagation of L-BSE in sheep seemed to result in a TSE with a different profile to that of C-BSE (Nonno et al., 2008; Nicot et al., 2014). L-BSE isolates transmitted to transgenic mice expressing ovine PrP^C (Beringue et al., 2007), or inbred wild-type mouse lines (Capobianco et al.,

2007), acquired a phenotype indistinguishable from the BSE agent. However, the inoculation of tissues collected from mice over-expressing ovine PrP^C, inoculated with C-BSE and L-BSE, to bovine PrP^C transgenic mice resulted into two different phenotypes specific to each agent, suggesting that the agents passaged in ovine PrP transgenic mice, although producing a similar signature in the brain, were actually different (Beringue et al., 2010).

Transmission of H-BSE isolates originating from France and Poland to bovine PrP transgenic mice has been reported. While in the majority of the cases the propagated TSE was different from C-BSE, C-BSE has emerged in a proportion of the mice inoculated with two distinct isolates (one from France and one from Poland) (Torres et al., 2011). Baron et al. (2011) also observed strain features similar to C-BSE after inoculation of conventional mice with H-BSE.

Together these data indicate that there may be an aetiological relationship between Atypical and Classical BSE.

Intracerebral inoculation of brain tissue from L-BSE-infected cattle to cynomolgus macaques induced a spongiform encephalopathy distinct in all its aspects (clinical, pathological and biochemical) from macaque BSE (Comoy et al., 2008). Incubation periods were shorter for L-BSE (23–25 months) than for C-BSE (38–40 months), suggesting that L-BSE may be more virulent than C-BSE for infecting primates. L-BSE was also transmissible to microcebus, with shorter incubations than C-BSE (Baron et al., 2008). Moreover, experiments demonstrated the transmissibility of L-BSE to macaques by the oral route (Comoy, 2010). L-BSE was transmitted with success also to mouse lemurs, another primate model, after both i.c. and oral inoculation, with a longer incubation period and less severe clinical symptoms following oral challenge (Mestre-Francés et al., 2012).

Histology and biochemistry studies showed similarities between L-BSE-inoculated macaques and MM2 sporadic Creutzfeldt–Jakob disease (sCJD) patients: infected primates and those rare patients exhibited similar lesional profiles, and the disease-specific PrP from both groups showed the same N-terminal protease sensitivity. Moreover, a macaque inoculated with brain tissue from a MM2 sCJD patient showed a similar lesion profile to L-BSE-infected macaques (Comoy et al., 2009). Similarly, transmission of L-BSE into bank voles resulted in a TSE with phenotypic characteristics (incubation period, PrP^{Sc} biochemical properties and vacuolar lesion profiles) indistinguishable from those observed after transmission of a VV2 sCJD case in this rodent model (Nonno et al., 2009).

The i.c. inoculation of L-BSE field isolates produced TSE disease in two lines of mice over-expressing human PrP (Met129), exhibiting a molecular phenotype distinct from that of C-BSE (Beringue et al., 2008a; Kong et al., 2008). In one of them, the L-BSE agent appeared to propagate with no obvious transmission barrier: a 100 % attack rate was observed on first passage, the incubation time was not reduced on subsequent passaging (Beringue et al., 2008a) and the L-BSE PrP^{Sc} biochemical signature was essentially conserved (Beringue et al., 2008a; Kong et al., 2008), appearing to be indistinguishable from that seen after experimental inoculation of MM2 sCJD in these mice (Beringue et al., 2007). These transmission features markedly differed from the low transmission efficiency of cattle C-BSE isolates to this (Beringue et al., 2008a; Beringue et al., 2008b) and other (Asante et al., 2002; Wilson et al., 2012) human PrP transgenic mouse lines, based on the presence of clinical signs and/or protease-resistant prion protein (PrP^{res}) in the brain (Beringue et al., 2012).

H-BSE isolates failed to infect one line of “humanised” mice (Met129 PrP) (Beringue et al., 2008a). These mice over-express human PrP and were inoculated i.c. with a low dilution inoculum, supporting the view that the transmission barrier of H-BSE from cattle to humans (expressing this allele) might be quite robust.

The permissiveness to Atypical BSEs of “humanised” transgenic mice expressing the valine allele at codon 129 is currently unknown.

1.4. Detection of infection

Limited data are available on the performance of the validated rapid tests used for cattle TSE testing for the detection of Atypical BSE cases, in terms of either their analytical sensitivity or their ability to detect infected asymptomatic animals. This results in uncertainty about the true prevalence of these conditions. However, Meloni et al. (2012) compared the analytical sensitivity of EU-approved commercial TSE rapid tests for C-BSE, H-BSE and L-BSE, and, although some differences in the analytical sensitivity were reported, all tests succeeded in detecting the three BSE forms at a 1:16 dilution prepared following the manufacturers' instructions.

In C-BSE cases, pathogenesis studies have established that abnormal PrP deposition in the brainstem first occurs at the obex level, where substantial amounts of this disease-specific protein accumulate during the late incubation phase (Arnold et al., 2007; Wells et al., 2007; Simmons et al., 2010). As a consequence, targeting of the brainstem at the level of the obex for C-BSE rapid testing is considered to be the most sensitive approach for detecting cases within the framework of the active surveillance system.

In Atypical BSE (both H- and L-BSE), the suitability of the obex as the target tissue for testing that would allow an early and sensitive detection of these conditions remains questionable, although all the Atypical BSE cases detected so far have been identified through the active surveillance system, indicating that obex testing with currently validated tests allows the detection of at least a proportion of these cases.

When discriminating the different forms of BSE, immunohistochemistry (IHC) in brain has been reported to allow discrimination of distinguishable immunolabelling patterns for C-BSE, L-BSE and H-BSE, especially when focusing on rostral areas of the brain, and cerebellum (Konold et al., 2012). Western blotting (WB) allows prompt identification of H-BSE cases according to the molecular mass of the unglycosylated PrP fraction and the detection of the protein by antibodies binding to N-terminal epitopes (e.g. SAF32, P4); L-BSE can be identified by the lower molecular mass of the unglycosylated PrP fraction and the altered glycoprofile (similar quantities of di- and monoglycosylated PrP). This WB classification is the method currently stipulated in the EU TSE Regulation.

1.5. Concluding remarks

- Data relating to the prevalence and geographical distribution of Atypical BSE are incomplete and subject to variation owing to the ongoing retrospective typing of BSE cases.
- All Atypical BSE cases have been detected by active surveillance, typically in animals over eight years of age, with a similar number of cases detected each year from 2001 to 2013.
- The recent cessation of the testing of healthy slaughtered cattle in some EU Member States will lead to a loss of capacity of the monitoring system to detect Atypical BSE cases.
- Sampling and rapid testing procedures, as currently performed, have not been formally evaluated for the detection of Atypical BSE and might have an impact on the capacity to monitor the epidemiological situation of the disease.
- Information on the pathogenesis and tissue distribution of Atypical BSE in cattle through the study of field cases and experimental transmission studies is lacking. The latter are limited to transmission of Atypical BSE through i.c. inoculation of cattle.
- For transmission studies, i.c. challenge would be an appropriate proxy for studying the distribution of the agent if the origin of the disease was spontaneous, and originating in the brain, while oral challenge would be more appropriate if the origin of the disease was through ingestion of infected material.
- The current lack of information on the distribution of infectivity in tissues from Atypical BSE-infected cattle does not allow judgement of whether the current list of bovine SRM, set by EU

legislation based on data relating to the pathogenesis and tissue distribution of C-BSE, is fit for the purpose of removing most of the Atypical BSE infectivity from bovine carcasses.

- As is the case for C-BSE, Atypical BSE (H-BSE and/or L-BSE) agents are able to propagate in experimentally challenged foreign species such as mice, sheep, voles, primates and hamsters, and in transgenic mice expressing heterologous, i.e. non-bovine, PrP sequences.

2. Biological material collected from field cases and transmission studies in cattle

2.1. Information available from published literature

Intracerebral transmission studies in cattle with both Atypical BSE forms have been performed at different institutes. Lombardi et al. (2008) described the i.c. challenge of six cattle with L-BSE and, as a control, six cattle with C-BSE. The clinical signs were described and the PrP^{Sc} distribution in different brain regions and the spinal cord were analysed. A follow-up study by Suardi et al. (2012) reported on the presence of BSE infectivity and PrP^{Sc} deposition in peripheral muscle samples taken from these L-BSE challenged cattle.

A similar study was performed in Japan, in which the first Japanese L-BSE isolate was used for an i.c. challenge experiment in five cattle (Fukuda et al., 2009). It could be shown by WB analysis that numerous peripheral nervous tissues contained PrP^{Sc} (Iwamaru et al., 2010). In contrast, all samples from the lymphoreticular system turned out to be negative. A similar study was performed by the same group using a Canadian H-BSE isolate for the i.c. challenge of three cattle. As already shown for L-BSE, the presence of PrP^{Sc} in various peripheral nervous tissues could be determined (Okada et al., 2011b).

In a different i.c. challenge study, five cattle were inoculated with L-BSE and six cattle with H-BSE (Balkema-Buschmann et al., 2011). The general restriction to the central nervous system (CNS) that had already been described for C-BSE was confirmed in these cattle.

Intracerebral challenge studies with both Atypical BSE forms were also performed by Konold et al. (2012), in which PrP^{Sc} deposition was identified in muscle spindles and in the trigeminal ganglion following a limited study of potential peripheral distribution. This study is described in more detail in Section 2.2.

There have also been a few field cases of L-BSE in which more extensive examination and collection of peripheral tissues was possible (Mazza et al., 2007; Suardi et al., 2012).

2.2. The European Union Reference Laboratory (EURL) study

The majority of Atypical BSE cases so far identified worldwide have been detected by active surveillance. Consequently, the volume and quality of material available for the underpinning of confirmatory diagnosis and classification and the demonstration of robust statutory testing through external quality assessment (EQA) was very limited. This small cattle transmission study, of both H- and L-BSE, had the primary aim of providing tissue for test evaluation and research, and to generate clinical, molecular and pathological data in a standardised way to enable more robust comparison of the two variants with particular reference to those aspects most relevant to case ascertainment and diagnosis within existing regulated surveillance programmes.

In the first study (Konold et al., 2012), groups of four calves (10–11 months of age) were intracerebrally inoculated with 1.0 mL of either L-BSE or H-BSE brain homogenate (in 10 % w/v solution in sterile saline) prepared from a French cow with H-BSE (code: ESB-H-07-0644) and an Italian cow with L-BSE (code: 141387/02). Prior to inoculation, the inocula were either heat treated (twice for 15 minutes at 70 °C; H-BSE) or treated with antibiotics (L-BSE) according to established methods (Wells and Hawkins, 2004).

The animals were kept until clinical signs developed, and these clinical signs were systematically monitored and described. All the animals presented with a nervous disease with some similarities to C-BSE, which progressed to a more dull form in one animal from each group. Difficulty rising was a consistent feature of both disease forms and was not seen in two BSE-free, non-inoculated cattle that served as controls. The animals were then killed and the brains removed to fulfil the primary tissue generation objective of the study. Limited pathology and molecular characterisation was undertaken to ensure that the diagnostic features of the experimentally generated material were representative of the original field case donor animals. A range of additional peripheral tissues (neural and non-neural) were taken, to generate matched tissue sets with material previously collected from animals challenged in a similar way with scrapie (Konold et al., 2006). These tissue sets were stored in the Defra TSE Archive for possible future research requests. A complete list of tissues collected and description of analyses already performed are provided in Table 3 (see also Appendix A).

Following the requirement for all bovine TSE isolates arising within the EU to be classified, a further four animals (two H-BSE and two L-BSE) were challenged intracerebrally to augment the material available to the EURL for EQA and provision of appropriate controls for diagnostic investigations. These challenges were sub-passages of some of the primary challenge cases. Experimental donor material was chosen partly because of its provenance, with regard to relative microbiological stability and clinical status, and partly to establish that the phenotype of the disease remained stable following experimental passage.

Each challenge was a single donor to a single recipient design, and the donors were chosen based on the greatest dissimilarity of clinical presentation in the primary challenges (i.e. one animal from each group which had shown a nervous clinical presentation and one with a dull presentation). All inocula were tested for bacterial contamination and treated with antibiotics if required. The recipient calves were seven months old at challenge. As for the primary challenges, animals were monitored until the development and description of clinical disease, at which point the animals were killed and the same tissue collection and disease characterisation protocols were applied.

Part of this characterisation was the phenotypic description, but not titration, of the H- and L-BSE material in bovinised transgenic mice (tg110) both before and after each experimental passage. Some of these bioassays are still ongoing.

Table 3: Material collected within the EURL study and related information on conservation status and testing performed

Tissue	Status of material available		Testing performed
	Fixed	Frozen	
Central nervous system			
Brain	x	x	IHC and WB positive
Spinal cord	x	x	IHC positive
Peripheral nervous system - ganglia and plexi			
Cranial cervical ganglion	x		
Nodose ganglion	x		
Stellate ganglion	x		
Trigeminal ganglion	x	x	IHC positive
Peripheral nervous system - nerves			
Facial nerve	x		
Radial nerve	x		
Sciatic nerve	x	x	
Splanchnic nerve	x		
Sympathetic chain	x		
Vago-sympathetic trunk	x		
Vagus nerve	x		
Lymphoid tissue			
Bronchomediastinal LN	x	x	
Cervical thymus	x	x	
Hepatic LN	x	x	
Lateral retropharyngeal LN	x	x	
Medial retropharyngeal LN	x	x	IHC negative
Mesenteric LN	x	x	IHC negative
Nictitating membrane	x		
Palatine tonsil	x	x	IHC negative
Pharyngeal tonsil	x		
Popliteal LN	x	x	
Prefemoral LN	x	x	
Prescapular LN	x	x	
Submandibular LN	x	x	
Spleen	x	x	
Gastrointestinal tissues			
Cranial oesophagus	x	x	
Distal ileum (Peyer's patches)	x	x	IHC negative
Jejunum (Peyer's patches)	x	x	
Muscles			
Extra-ocular muscles	x	x	IHC positive
Medial gluteal muscle	x	x	IHC positive
Semitendinosus muscle	x	x	
Triceps muscle	x	x	IHC positive
Other tissues			
Adrenal gland	x	x	
Eye	x	x	
Kidney	x	x	
Parotid salivary gland	x	x	
Sub-mandibular salivary gland	x	x	
Body fluids			
Blood fractions		x	
Cerebrospinal fluid		x	

LN, lymph nodes.

2.3. Additional information on material available

Data available from the literature relating to field case examinations, the EURL study and the other transmission studies outlined above (Section 2.1) have been collated to provide a comprehensive list of the tissue samples that have been collected and examined from natural and experimental cases of H-BSE and L-BSE. The full list is given in Appendix A and represents a wide range of tissues from both the central and peripheral nervous systems, the lymphoreticular system, the musculoskeletal system and the gastrointestinal tract, together with other principal edible organs. However, none of these studies was explicitly designed to address the issue of potential infectivity in the context of the current legislation on SRM, and so there are some SRM tissues that have not been collected within any of these studies, namely the duodenum, the jejunum and ileum (without Peyer's patches), the caecum, the colon and the mesenteric fat. For these tissues there are therefore no data, and no possibility to create data without undertaking further experimental challenges. All of the experimental material is derived from animals that have been challenged intracerebrally.

In total, data are available for 15 experimental H-BSE and 23 L-BSE cases, representing challenges with 4 donors respectively, while data on PrP distribution in naturally-occurring field cases have been published for only three L-BSE-affected cattle.

There are no data for field case H-BSE. Where data exist from both field cases and experimental animals (i.e. for L-BSE only), there is good agreement between the data with and abnormal PrP distribution, with the CNS and muscles both consistently affected. However, these data relate to the presence or absence of PrP^{Sc}, and do not attempt to quantify relative amounts of PrP^{Sc} or levels of infectivity.

Overall, disease-related PrP has been reported in CNS tissues, peripheral ganglia and nerves, muscles (predominantly muscle spindles), adrenal glands and retina for both H-BSE and L-BSE. By contrast, no lymphoid tissues or gastrointestinal tissues have tested positive by IHC, WB or bioassay. Some tissues have been collected, but with no testing outcome explicitly reported.

Comparative data relating to C-BSE (see Appendix A, Table 12) are to a large extent fragmented, and have evolved over a long period as different analytical methods have been developed and applied. Many of the original infectivity data are based on conventional mouse bioassay rather than transgenic models so, although positive results are robust, negative results do not necessarily have the same sensitivity thresholds (see also EFSA BIOHAZ Panel (2014)). It must also be noted that data available for C-BSE are derived predominantly from field cases or oral challenge models. There are few data from i.c. challenge studies that can be directly compared with existing data for Atypical BSE.

However, collective data indicate that C-BSE shares the same tissue distribution as the Atypical BSE cases, with PrP^{Sc} and/or infectivity detected in the central and peripheral nervous systems, including ganglia and nerves, and the muscle spindles in skeletal muscle. As in Atypical BSE, the adrenal glands and the retina are also affected.

Additionally, in C-BSE there is also evidence of involvement of the lymphoreticular system, particularly, but not exclusively, in those tissues associated with the gastrointestinal tract. The PrP^{Sc} distribution and relative levels of infectivity in the gastrointestinal tissues were presented in detail in a previous EFSA Opinion (EFSA BIOHAZ Panel, 2014). Nasal mucosa and bone marrow have also been shown to contain infectivity.

There are insufficient data at present to be clear about whether these apparent differences in the distribution of disease-specific markers reflect absolute differences between C-BSE and the H-BSE and L-BSE variants, whether they are a consequence of detection threshold limitations, or whether they are a consequence of the different routes of challenge.

2.4. Concluding remarks

- Where data exist from both field cases and experimental animals (i.e. for L-BSE only), there is good agreement of the data with regard to abnormal PrP distribution. There are no data for field case H-BSE.
- All data currently available relate to the presence or absence of PrP^{Sc}, but do not quantify relative amounts of PrP^{Sc} or levels of infectivity.
- Disease-related PrP has been reported consistently in CNS tissues, peripheral ganglia and nerves, muscles (predominantly muscle spindles), adrenal glands and retina for both H-BSE and L-BSE. All of these tissues are also positive in C-BSE.
- By contrast with C-BSE, at this stage no lymphoid tissues or gastrointestinal tissues from H-BSE- and L-BSE-affected animals have tested positive for PrP^{Sc} presence (IHC, WB) or infectivity (bioassay).
- There are insufficient data at present to be clear about whether these apparent differences in the distribution of disease-specific markers reflect absolute differences between C-BSE and the H-BSE and L-BSE variants, whether they are a consequence of detection threshold limitations, or whether they are a consequence of the different routes of challenge.
- No studies have been explicitly designed to address the issue of Atypical BSE with respect to SRM regulations. Without further experimental challenges or tissue collection from ongoing studies it will not be possible to obtain any data on duodenum, the jejunum and ileum (without Peyer's patches), the caecum, the colon and the mesenteric fat.

3. Generic approach to quantify Atypical BSE prions in cattle tissues

3.1. Bioassay

Estimation of a TSE agent's infectivity in tissues is traditionally determined by bioassay. This involves the experimental inoculation of groups of animals either by the i.c. route only or by both the intraperitoneal (i.p.) and the i.c. route and the recording of the resulting disease attack rate, incubation period and the percentage of inoculated animals that develop clinical signs and/or accumulate detectable levels of abnormal prion protein in brain or peripheral tissues, to estimate the infectious titre.

3.1.1. Choice of the mouse model

The development of transgenic mouse models for the PrP gene allowed the species barrier (a phenomenon that limits TSE agent primary passage in conventional rodent models) to be reduced/suppressed. PrP sequence identity between the transgenic host and donor are usually associated with a higher transmission rate than in wild-type mice. A clear increase in transmission rate for the three BSE agents (C-BSE, L-BSE and H-BSE) was observed upon transmission in a gene targeted transgenic mouse line expressing the bovine prion protein (Bov6) when compared with a wild-type control of the same genetic background (Wilson et al., 2013). However, the low PrP expression level of these gene-targeted Tgbov mouse models represents a major handicap for their use in prion detection. In fact, the long incubation times and the partial transmission rates in these mice inoculated with the three BSE agents highlight the low utility of these models for the detection of these agents.

Several mouse lines over-expressing bovine PrPC have been developed: Tg(BoPrP) 4092HOZ (Scott et al., 1997), BoPrP-Tg110 (Castilla et al., 2003), Tgbov XV (Buschmann and Groschup, 2005), and TgBo-Tg540 (Beringue et al., 2006), and have shown good sensitivity for the detection of bovine prion infectivity. Several studies have shown that all three BSE agents are efficiently transmitted to these transgenic mice, showing neuropathological and molecular phenotypes that are distinct for each BSE agent (Beringue et al., 2006; Buschmann et al., 2006; Beringue et al., 2007; Capobianco et al., 2007; Okada et al., 2010; Torres et al., 2011). In all cases, all three BSE agents were fully transmitted

to these Tg-bov mouse models, although L-BSE showed survival times that were shorter (≈ 200 days) than H-BSE (≈ 250–300 days), but which were higher than those of C-BSE.

Table 4 summarises transmission data available for each mouse model when inoculated with the three BSE agents. Although the isolates used were not the same for each mouse model, these data suggest that any of these mouse lines over-expressing bovine PrP are adequate for the detection of both L-BSE and H-BSE.

Because the interspecies transmission of prion infection is essentially governed by the structural compatibility between recipient host PrP^C and the infecting PrP^{Sc} strain type(s) (Collinge and Clarke, 2007), non-bovine PrP transgenic mouse lines or species might, in principle, be able to propagate bovine BSE prions without any apparent species barrier. This has been exemplified by the high susceptibility of the bank vole to sCJD (Nonno et al., 2006).

Table 4: Survival times (days) and transmission rates (%) of BSE agents in several mouse models

Mouse line	C-BSE	L-BSE	H-BSE	Reference
TgBo110	250–300 (100 %)	190–220 (100 %)	280–350 (100 %)	Torres et al. (2011) (see also Appendix A)
Tg(BoPrP) 4092HOZ/$Prnp^{0/0}$	243 ± 7 (100 %)	198 ± 34[a] (100 %)	316 ± 12 (100 %)	Okada et al. (2011a); Masujin (2008)
Tgbov XV	250–300 (100 %)	200–215 (100 %)	322 (100 %)	Buschmann et al. (2006); Capobianco et al. (2007)
TgBo-540	300–380 (100 %)	210–230 (100 %)	400–415 (100 %)	Beringue et al. (2006); Beringue et al. (2007)
Bo6 (knock-in mice)	518 ± 18 (15/22)	547 ± 18 (2/24)	561 ± 15 (1/23)	Wilson et al. (2012)
129/Ola (wild-type mice)	498 ± 43 (8/8)	NA (0/24)	NA (0/24)	Wilson et al. (2012)

NA: no mice showed clinical signs of disease.

3.1.2. Bioassay results and infectious titre estimates

When trying to estimate the infectious titre of a TSE agent in a sample using bioassay transmission results, several approaches can be used.

3.1.2.1. Endpoint titration approach

The most straightforward and accurate approach to estimate the infectious titre in prion diseases is the Spearman–Karber method. In this approach, groups of animals are challenged with successive dilutions of an inoculum and studied for the development of the disease and/or presence of detectable levels of PrP^{Sc} in brain or peripheral tissues. Infectious titre is assessed by the Spearman–Karber formula (Hamilton et al., 1977) and expressed as LD_{50} (lethal dose) or ID_{50} (infectious dose) per gram of inoculated tissue (where one LD_{50} or one ID_{50} corresponds to the amount of substance required to kill/infect 50 % of challenged animals).

Spearman–Karber formula: $\log_{10}LD_{50} = (X_0 - [d/2]) + d(\Sigma r_i/n_i)$, where:

X_0 = $\log_{10}$ of the highest dilution at which all mice died of the disease

d = dilution factor

r_i = number of positive mice in the X_0 and subsequent dilutions

n_i = number of mice inoculated in the X_0 and subsequent dilutions (discounting those dying from intercurrent deaths but taking into account those whose brains contained PrP^{Sc} but did not develop symptomatic disease)

The model provides a maximum likelihood infectious titre (LD_{50} or ID_{50}) and a confidence interval (CI) (Markus et al., 1995). However, to provide an accurate estimate of the infectious titre, the Spearman–Karber method requires that at least one of the tested dilutions displays a 100 % attack or infection rate. This requirement makes this approach unsuitable for estimating titres in samples containing low infectivity.

3.1.2.2. Incubation period/attack rate derived models

Although regarded as less accurate than the endpoint titration approach, dose–response relationships have been used as a method for infectivity estimation (Prusiner et al., 1982; Heikenwalder et al., 2007; Lacroux et al., 2008) when endpoint titration data are not available.

For a given TSE agent (strain), the approach relies on:

- the endpoint titration of a reference inoculum;
- the computation of a dose-response curve reporting for a given incubation period the associated infectious titre of the inoculum; and
- the use of this dose–response curve to estimate the infectious titre of other inocula on the basis of their inoculation in the reference animal model.

More recently, Arnold et al. (2009) developed a method able to provide a more accurate estimation of the infectious titre than using attack rate alone and providing a natural way to deal with experimental animals that have died before the end of the experiment without having to decide debatable inclusion/exclusion criteria. The titre of infectivity is estimated using both the probability of survival (attack rate at each dilution) and the individual mouse incubation periods at each dilution using a Bayesian approach instead of the maximum likelihood approach.

The method considers not only the incubation period observed in mice but also the possibility that an infected mouse could die from causes unrelated to BSE (e.g. intercurrent disease or sacrifice at the experimental endpoint) during the experiment.

3.1.2.3. Poisson distribution-derived models

The use of the Poisson distribution to calculate the resulting titres and standard deviations in TSE agent inocula was initially used in the prion field to estimate the infectious titre in blood fractions prepared from infected mice (Brown et al., 1998). The complete methodology is described in an appendix to the paper of Brown et al. (1999). The approach relies on the principle of the random distribution of physical particles in low-concentration solutions. It is based on the attack rate/transmission rate observed following inoculation of a known volume of inoculum to a group of animals and it does not require any reference (dose–response) to provide a titre estimate. The method has been extensively employed for measurements of infectivity in blood, blood components and urine of TSE-infected rodents (under the denomination “Limiting Dilution Titration”). This statistical approach can be applied to inocula which do not fulfil the criteria for the Spearman–Karber method application (at least one dilution infecting/killing 100 % of the animals).

This approach also provides an estimate of the maximum infectious titre (upper boundary CI 95 %) that can be found in an inoculum when no transmission is observed in a bioassay experiment (Brown et al., 1999; Andreoletti et al., 2012).

The Poisson calculation returns a value for the ID that can be converted into ID_{50} (Fisher, 1936; Gregori et al., 2006).

3.1.3. Overall considerations

- Low, if any, infectivity is expected to be found in many of the cattle tissues. In this context, incubation period/attack rate and Poisson distribution-derived models appear to be the most suitable approaches for estimating the infectious titres in these tissues.
- Estimate of maximum infectious titre in negatively bioassayed tissues:
  - The amount of prion infectivity that is below the threshold of detectability of a mouse bioassay but that might be present in the studied tissue can be estimated in infectious units (IU) per millilitre with 95 % CI (rather than in 50 % infectious dose (ID_{50})) by assuming a Poisson distribution in the response variable, as proposed by Notari et al. (2012).
  - More recently, Makarava et al. (2012) also proposed the use of the Poisson equation for determining prion infectivity which is present below the limit of detectability, although infectivity was expressed in ID_{50} (Makarava et al., 2012). This way, for the probability that a given inoculum dose contains no active particle, $P(0)$, the Poisson equation reduces to:

    $$P(0) = e^{-m}$$

    where m is the concentration of the active particle at that dilution in particles per inoculation volume.

    The probability that there will be at least one active particle to initiate infection, $P(\geq 1)$, is:

    $$P(\geq 1) = 1 - e^{-m}$$

    Infectivity data can be fitted to the expression:

    $$F = 1 - \exp(-ax)$$

    where F is the fraction of infected animals, x is the dilution and a is the fitted parameter equal to the undiluted concentration of infectivity.

    ID_{50} can be calculated by solving the fitted equations for $F = 0.5$ (infectivity), which reduce to the expression:

    $$ID_{50} = -\ln(0.5)/a$$

3.2. *In vitro* amplification

3.2.1. *In vitro* amplification techniques

Two very efficient procedures to amplify prions in a test tube have emerged in the last decade. Both rely on the detection of PrP structural conversion and polymerisation upon addition of PrP^{Sc} "seeds" contained in the infected samples (for a review see Kraus et al. (2013)).

The protein misfolding cyclic amplification (PMCA) assay is based on repetitive cycles of incubation and sonication, using a PrP^{C}-containing substrate (in excess). Incubation of PrP^{Sc} seeds with PrP^{C}-containing substrate is thought to favour PrP^{C} conversion and growth of PrP^{Sc} aggregates. Sonication is thought to fragment the polymers, thus multiplying the number of seeds for further conversion. The cyclic nature of the system and the possibility to refresh the substrate at each round enables the performance of as many cycles as required to reach the amplification state needed for the detection of PrP^{Sc} in a given sample. Monitoring is performed by conventional Western blot or enzyme-linked immunosorbent assay (ELISA)-based techniques. PMCA allows the detection of minute amounts of PrP^{Sc} in biological tissues or fluid samples including blood, urine, faeces or cerebrospinal fluid from many prion-infected species (Castilla et al., 2005; Kurt et al., 2007; Lacroux et al., 2012).

The quaking-induced conversion (QuIC) and amyloid seeding assay (ASA) monitor the conformational conversion of soluble, alpha-helix-rich recombinant PrP (recPrP) into beta-sheet-enriched amyloid-like fibrils following the addition of PrP^{Sc} (for a review see Orru et al. (2012)). Shaking instead of sonication is performed to break the generated polymers and provide new seeds for conversion. In its real-time, high-throughput format (RT-QuIC), prion-seeded amyloid formation of recPrP is followed by detection of thioflavin T (ThT) fluorescence over time. recPrP substrate replacement has been reported to dramatically improve the QuIC sensitivity (Orru et al., 2011). The technique has proved sensitive in detecting prions in several infected tissues and in fluids such as cerebrospinal fluid, saliva, nasal fluids and blood (Safar et al., 2008; Atarashi et al., 2011; Orru et al., 2011; McGuire et al., 2012).

3.2.2. Definition of the quantification approach of seeding activity

These methods do not detect prion infectivity in a given tissue, but allow detection of a seeding activity potentially associated with prion replication. They can thus be considered on first intention as surrogate markers of prion infectivity (or prion seeding activity). Under defined conditions, these methods can be used to quantitatively estimate prion concentration in fluids and tissues of interest.

In most PMCA protocols (Castilla et al., 2005), there is a direct relationship between the amount of PrP^{Sc} in the sample and the number of PMCA rounds necessary for detection. For quantification, the amplified PrP^{Sc} signal can be compared with that seen in endpoint titrated material run in the same conditions (such as brain homogenate from animals at the terminal stage of disease) or to PrP calibration curves (Chen et al., 2010).

By analogy with animal bioassays, both PMCA and RT-QuIC assays can titrate the seeding activity in endpoint diluted samples (Wilham et al., 2010; Makarava et al., 2012). For PMCA, this can be done over several rounds (Makarava et al., 2012) or in several independent experiments when maximal sensitivity is achieved within a round, i.e. without substrate refreshment (Moudjou et al., 2013). Provided a sufficient number of replicates are performed (see below), it allows the establishment of a “seeding dose” giving positive responses in 50 % of replicate reaction (SD_{50}).

3.2.3. Choice of the substrate

In both PMCA and QuIC methods, the choice of the PrP substrate is a critical parameter for achieving efficient and sensitive amplification.

For PMCA, the nature of the tissue that serves as the PrP substrate (brain, extraneural tissue, cells, etc.), the genetic background (notably for transgenic mouse lines expressing the PrP of interest), the primary structure of PrP^{C} and the level of PrP^{C} in the substrate have all been shown to strongly modulate PMCA sensitivity by several orders of magnitude (Mays et al., 2009; Mays et al., 2011; Moudjou et al., 2013).

For QuIC, the conformational diversity of recombinant PrP from different animal species used so far as a substrate in QuIC assays remains poorly investigated. Hamster, human, chimeric human–hamster or chimeric sheep–hamster recPrP constructs are mostly used in the different assays reported, independently of the prion strain and species to be amplified (Atarashi et al., 2007; Atarashi et al., 2011; Orru et al., 2011; McGuire et al., 2012).

For both methods, the best PrP substrate may not be the one that is the most homologous (with regard to PrP primary sequence) to the type of prion to be assayed.

3.2.4. Repeatability and robustness

Owing to the inconsistent amplification/fibrilisation observed in reactions seeded with highly diluted material, performing at least four or five replicates per sample and dilution is a critical parameter when assessing the minimal seeding activity in homogenates (Castilla et al., 2005; Makarava et al., 2012).

3.2.5. Availability of negative samples

Because of the sensitivity achieved with the cell-free techniques, sometimes at the sub-infectious level, the possibility of false-positive results due to cross-contamination is high (Cosseddu et al., 2011). A significant number of negative samples must be tested along with the positive ones to exclude any cross-contamination. If there is any evidence of cross-contamination, the whole run must be discarded.

Negative controls are also important to ensure that any potential inhibitory impact of the substrate of the tissue to be assayed is established.

3.2.6. Caveats and limitations

It is not known at this stage whether Atypical BSE prions are amplifiable by cell-free methods. Some prion strain types are known to be fairly resistant to amplification by either PMCA or QuIC.

Concentrated tissues or fluid components can also interfere, notably with QuIC amplification (Wilham et al., 2010), sometimes necessitating the use of a PrP^{Sc} (semi)purification step (Orru et al., 2011; Segarra et al., 2013). Blood as a substrate is known to be an issue, making it difficult to apply these techniques directly to such samples (Castilla et al., 2005; Lacroux et al., 2014).

Regarding specifically the PMCA technique, the necessity, so far, to use brain tissue homogenate as a substrate in order to achieve high amplification levels constitutes a limitation. There are also significant variations in the laboratory-specific methodologies employed to amplify PrP^{Sc}, resulting in discrepancies or variations in the reproducibility of experiments. Finally, the probability of false-positives increases as the number of rounds increases (Cosseddu et al., 2011).

Regarding the QuIC/ASA methods, a confounding aspect is the frequent spontaneous formation of recPrP fibrils (without seeding or with seeding at very low concentration) over time, and sometimes soon after seeding, which reduces the test specificity (Colby et al., 2007).

3.3. Correlation of sensitivity between *in vitro* amplification and bioassay

A number of studies have directly compared the performances of cell-free and animal bioassays in determining endpoint titrations (Saa et al., 2006; Wilham et al., 2010; Johnson et al., 2012; Makarava et al., 2012; Moudjou et al., 2013). The sensitivity achieved is generally superior to that of the bioassay by two to three orders of magnitude (Saa et al., 2006; Johnson et al., 2012; Makarava et al., 2012; Moudjou et al., 2013).

This difference has been attributed to a large excess of reactive prion protein seeds with little or no infectivity or, alternatively, to the higher rate of clearance of PrP^{Sc} seeds in animals versus cell-free assay reactions.

Interestingly, while there was a good fit to the Poisson equation for animal infectivity data for two TSE agents in hamster (263K and SSLOW) at limiting dilution, the PMCA titration curves for both strains showed a more gradual slope than predicted by the Poisson equation, which might be explained by the increasing PMCA reaction efficiency at higher dilutions or the fact that inoculum dilution results in a concentration-dependent dissociation of aggregates, thereby releasing and increasing the concentration of PMCA reactive centres (Makarava et al., 2012). Despite these discrepancies, well-calibrated PMCA reactions can be an efficient and cost-effective method for the estimation of PrP^{Sc} titre, at least for certain strains.

3.4. Concluding remarks

- The reference method for the estimation of prion infectious titre in tissues is endpoint dilution titration in animals. To achieve maximum sensitivity regarding Atypical H-BSE and L-BSE,

this bioassay should ideally be done in mouse lines over-expressing bovine PrP^C. Several mouse lines over-expressing bovine PrP^C are available worldwide.

- Several approaches can be used to estimate the infectious titre in a sample using a mouse bioassay, depending on the level of infectivity expected in that sample:
  - In samples containing high and moderate infectivity the most straightforward and accurate approach is endpoint titration by the Spearman–Karber method. Although regarded as less accurate than the endpoint titration approach, dose–response relationships can be used as a method for infectivity estimation when endpoint titration data are not available, provided an endpoint titration of a reference inoculum is provided in parallel.
  - In the context of a low infectious titre, incubation period/attack rate and Poisson distribution-derived models are suitable for estimating the infectious titres.
  - The amount of prion infectivity that is below the threshold of detectability of mouse bioassay but that might be present in the studied tissue can be estimated by assuming a Poisson distribution in the response variable.
- *In vitro* amplification techniques can be used to determine whether a tissue contains any prion seeding activity.
- These methods are usually more sensitive than the bioassay, and endpoint titration can be performed in a format similar to the bioassay.
- Given the current uncertainties in the specificity of these techniques and the absence of standardisation between laboratories, it is necessary to provide ad-hoc control samples and to replicate the experiments to achieve statistical significance.
- A correlation must be made between the sensitivity achieved by the cell-free assays and the bioassays using reference material such as brain tissue from animals at the terminal stage of disease.

4. Recommended approach to establish a laboratory protocol to investigate presence, distribution and infectivity level of Atypical BSE

The present protocol intends to provide a basis for studies to obtain information on:

- the distribution of H-BSE and L-BSE agents in the tissues of affected cattle, with special emphasis on tissues belonging to the SRM list;
- the relative amount of H-BSE and L-BSE agents that can be detected in tissues other than SRM.

In the framework of past evaluation of new rapid tests for the detection of TSE in ruminants (EFSA BIOHAZ Panel, 2012), EFSA developed a protocol for the evaluation of new rapid BSE post-mortem tests (EFSA, 2007). This protocol did not include evaluation of the performance of rapid tests with regard to Atypical BSE, since at the time no adequate material was available to perform this evaluation. However, it recommended this should be done once H- or L-BSE-infected brain material becomes available, and that, if rapid tests do not meet performance criteria on H- and L-BSE, they should not be considered for field testing. Therefore, in accordance with such recommendations, information should now be obtained on the performance of currently validated rapid tests for TSE active surveillance in cattle relative to bioassay for the detection of H-BSE and L-BSE agents.

4.1. Selection of tissues

4.1.1. BSE infectivity in bovine tissues

The current SRM list in cattle (see Table 5) has been established and amended in accordance with current knowledge related to C-BSE agent distribution in the tissues of infected cattle.

The SRM measure is aimed at preventing the entry into the food chain of tissues and anatomical structures that might contain significant amounts of infectivity. In the framework of C-BSE infection in cattle, these measures are extremely efficient. However, certain tissues that might contain low amounts of infectivity in certain BSE-infected cattle are not included in the cattle SRM list (Table 6).

Table 5: Bovine SRM list, as defined in Regulation (EC) No 999/2001

Bovine SRM list
The skull excluding the mandible and including the brain and eyes, and the spinal cord of animals aged over 12 months
The vertebral column excluding the vertebrae of the tail, the spinous and transverse processes of the cervical, thoracic and lumbar vertebrae and the median sacral crest and wings of the sacrum, but including the dorsal root ganglia, of animals aged over 30 months
The tonsils, the intestines from the duodenum to the rectum and the mesentery of animals of all ages

Table 6: Cattle tissues with known infectivity or PrP^{Sc} presence for C-BSE according to WHO (2010), and their inclusion or not in the current SRM list

	Higher-infectivity tissues	**Lower-infectivity tissues**
SRM	Brain	Nictitating membrane
	Dura mater	Autonomic ganglia
	Spinal cord	Tonsil
	Optic nerve	Ileum
	Retina	Jejunum
	Spinal ganglia	
	Trigeminal ganglia	
Non-SRM		Peripheral nerves
		Adrenal glands
		Bone marrow
		Skeletal muscle

All the TSE agents replicate and accumulate at a high level in the CNS and can disseminate (centrifugally and centripetally) along the peripheral (autonomic and motor) nervous system. However, in a given host the agent distribution and the relative level of infectivity in other tissues can vary substantially according to the TSE strain.

For instance, in humans, although sCJD infectivity is mostly confined to the CNS, numerous lymphoid organs have been shown to be infectious in patients affected with variant CJD (vCJD) (Gill et al., 2013).

Similarly, in sheep infected with Classical scrapie, a significant level of infectivity can be found in lymphoid organs (about 10^3 times less than in the same weight of brain tissue), whereas in animals affected with Atypical scrapie lymphoid organs contain either very low (about 10^7 times less than in the same weight of brain tissue) or no detectable infectivity (Andreoletti et al., 2011).

There are currently no quantitative data on the distribution of H-BSE and L-BSE in cattle tissues.

4.1.2. Prioritisation of tissues to be included in further studies

In order to assess the adequacy and the relative effectiveness of the current SRM measures for mitigating human dietary exposure to H-BSE and L-BSE, a large number of tissues/anatomical structures need to be tested for the presence of PrP^{Sc} and/or infectivity.

Considering the potential number of samples and the requirement for bioassay, the choice of tissues to be tested should be prioritised according to three criteria:

- the level of infectivity they contain in C-BSE-infected cattle (SRM list);
- the presence of infectivity, or PrP^{Sc} presence, demonstrated in Atypical BSEs or other TSEs in ruminants;
- the importance in terms of input into the food chain in the EU.

According to the above criteria, priority categories are defined in Table 7, which also indicates the availability of those tissues as a result of the EURL study.

The final decision regarding the testing of tissues belonging to the different tissue groups will depend on the feasibility (cost/number of bioassays) and final objectives of the study.

Table 7: Priority categories for tissues to be included in further studies, and availability of the tissues as a result of the EURL study

Priority 1	**Priority 2**	**Priority 3**
Cattle SRM list	**Non-SRM with low infectivity (or PrP^{Sc} presence) in ruminants and significant[(a)] input into the food chain**	**Non-SRM with low infectivity (or PrP^{Sc} presence) in ruminants and limited/no[(a)] input into the food chain**
Brain (>12 m)	Peripheral nerves (radial nerve[(b)], **sciatic nerve**, vagus nerve[(b)])	**Lymph nodes**
Spinal cord (> 12 months)	**Thymus**	**Spleen**
Autonomic ganglia	Abomasum	**Oesophagus**
Dorsal root ganglia (> 30 months)	Fore-stomach	**Adrenal gland**
Trigeminal ganglia (> 12 months)	**Skeletal muscle**	Mammary gland
Splanchnic nerve (mesentery)[(b)]	Tongue (muscular tissue)	Nasal mucosa
Nictitating membrane (> 12 months)[(b)]	Bone marrow	Pancreas
Tonsil	**Kidney**	Placenta
Adipose tissue (mesentery)	Liver	**Salivary gland**
Caecum	Milk	Blood (**whole blood, plasma,** red blood cells, **white blood cells**)
Colon		**Cerebrospinal fluid**
Duodenum		
Ileum (Peyer's patches)		
Ileum (other than Peyer's patches)		
Jejunum (Peyer's patches)		
Jejunum (other than Peyer's patches)		
Rectum		
Eyes (> 12 months)		

In bold: tissues collected from the EURL study, available as fixed and frozen material (frozen only for blood and cerebrospinal fluid) (see Table 3).
(a): Importance of tissues in terms of input in the food chain in the EU as qualitatively estimated by expert opinion.
(b): Tissues collected from the EURL study, but available only as fixed material (see Table 3).

4.2. Number of individuals to be tested

Considering the relatively low number of H-BSE- and L-BSE-infected cattle that are available, and the potential inter-individual variations that might exist in the distribution/amount of TSE agents in tissues, samples (as identified in Section 4.1.2) collected from at least three H-BSE- and three L-BSE-infected individuals should be tested.

It can be seen from Table 7 that not all the tissues listed were collected from the experimentally infected cattle. In order to obtain comprehensive data on all SRM tissues and other non-SRM tissues, material collected from other H-BSE and L-BSE cattle would have to be obtained from an alternative source, either through new experimental inoculation in cattle or from other tissue banks. Consequently, the brain of every animal that is used as a source of biological material for this protocol will have to be tested (PrP^{Sc} detection, bioassay endpoint titration, seeding activity).

4.3. Processing of tissues

In order to avoid false-positive results that might occur, in particular using PrP^{Sc} amplification methods and bioassay, samples should be collected, handled and prepared under strict TSE sterile conditions.

4.3.1. Preparation of samples

Except for the IHC that is carried out using formalin-fixed tissues, all other PrP detection tests and bioassay require the preparation of tissue homogenate.

The homogenisation procedure might affect the performance of certain rapid tests to detect positive samples. Consequently, for tissues that are to be tested by rapid tests (CNS) stock macerate (50 % weight volume in 5 % glucose solution) should be prepared, aliquoted and stored before processing to further experiments (see EFSA (2007)).

For other tissues, homogenisation (10 % weight/volume homogenate) should be carried in a 5 % glucose solution. Particular attention should be paid to the quality of homogenisation of material rich in connective tissue.

4.3.2. Identification of abnormal PrP

The PrP^{Sc} detection methods to be applied must display a sensitivity/specificity that is at least equivalent to that already demonstrated by the EURL.

4.3.2.1. Immunohistochemistry

The IHC protocol to be applied will have to be at least as sensitive as the method used by the EURL. If this needs to be established, the EURL will select blocks of brain tissue and skeletal muscle from three H-BSE- and three L-BSE-infected cattle that tested positive at the EURL, and brain tissue and skeletal muscle from three healthy cattle. The IHC protocol will have to correctly demonstrate at least the same extent of PrP^{Sc} immunolabelling in this panel of samples.

After this, PrP^{Sc} IHC detection should be systematically carried out on all the solid tissues listed in Table 7.

4.3.2.2. Western blotting

PrP^{Sc} WB will be applied to tissues according to the requirements defined in Section 4.4. Each sample will be tested in duplicate and in two different runs. Results will be considered positive when at the least two abnormal PrP bands (displaying equivalent electromobility to that observed in positive controls) are identified.

To ensure that the WB protocol to be applied is adequate, the EURL will prepare 1/10 dilution series of 10 % brain homogenates from three H-BSE- and three L-BSE-infected cattle. Dilutions will be carried out in healthy cattle brain homogenate. The dilution series will be aliquoted and stored at –80 °C by the EURL as reference material. Each dilution series will be tested by WB by the EURL using its in-house standard protocol. For each sample, the last positive dilution will be determined. Any method to be proposed for the protocol will have to demonstrate a sensitivity at least equivalent to that obtained by the EURL.

4.3.3. Quantification of seeding activity

Considering that the methodology to be applied (PMCA and QuIC) has not yet been subject to standardisation for testing in routine laboratories, it is not possible at this stage to provide a standard operating procedure for conducting this experiment. In particular, the laboratory(ies) in charge of the experiment will have to determine the nature of the substrate (brain homogenate from bovine transgenic mice for PMCA and recombinant protein for QuIC) and the amplification/fibrilisation conditions (temperature/sonication or agitation cycles) that are the most appropriate for conducting the experiment.

Nevertheless, certain recommendations can be provided for performing the experiment:

- For both L-BSE and H-BSE, the relative sensitivity of the seeding activity should first be established by comparing the results of the endpoint titration of brain homogenates by bioassay (see Section 4.4) with the last dilution of the same brain material (1/10 dilution series) able to provide amplification (PMCA) or fibrilisation of recombinant protein (QuIC).
- For each tissue, the seeding capacity should be established as the last dilution that is able to provide a PrP^{res} amplification (PMCA) or a fibrilisation of recombinant protein (QuIC).
- If PMCA is to be used, the samples should be amplified for as many rounds as are necessary to reach the amplification plateau without occurrence of any false-positive reaction (due to cross-contamination) in negative controls. PrP^{res}-positive reactions should be tested by WB to establish that the banding patterns display features that are typical of H-BSE or L-BSE.
- If QuIC is to be used, shaking/resting cycles should be continued until the occurrence of the first auto-fibrillation in the unseeded control reactions (negative control).
- In both PMCA and QuIC assays:
  - in each experiment, a positive control (brain dilution corresponding to the already established cut-off value of the assay) should be run to confirm that amplification or fibrilisation has occurred properly;
  - each sample should be tested in quadruplicate over two different runs.

4.3.4. Bioassay

Mouse bioassays should preferentially be carried out in over-expressing bovine PrP transgenic mice (Tgbov), which are considered to be highly efficient for the detection of bovine prions infectivity (see Section 3.1.1).

If different animal models are used for the bioassay, it should be demonstrated that they display a detection sensitivity for H-BSE and L-BSE equivalent to that observed in Tgbov. These results should include a comparative endpoint titration of the same dilution series for H-BSE and L-BSE cattle isolates in Tgbov and in the proposed alternative animal model.

Once the animal model is selected, bioassay testing should be by i.c. inoculation (20 µL per animal). Animals should be then be clinically monitored until the occurrence of TSE clinical signs, at which time they should be culled. The CNS and spleen samples should be individually collected and tested by WB. Half of the brain (including the midline) should be formalin fixed for IHC and vacuolar lesion profiling (Beck et al., 2010a; Beck et al., 2010b; Vickery et al., 2013). Animals without typical clinical signs should be euthanized at end of life and their brain and spleen tissues must be analyzed similarly.

Because of the potential thermosensitivity of H- and L-BSE agents, heating of the inocula prior to inoculation must be avoided.

4.4. Specific protocol for the three main categories of tissue

Depending on the type of tissue and the sampling procedure, certain tissues may only be available in smaller amounts. This may limit the type and the number of tests that can be carried out.

In addition, while substantial levels of TSE infectivity/PrP^{Sc} are likely to be present in certain tissues, other tissues are likely to have low or no prion content.

Table 8 provides a classification of tissues according to infectivity level and amount of tissue usually available. The protocol recommended for the three different categories of tissues is presented in sections 4.4.1 to 4.4.3 below.

Table 8: Classification of tissues according to infectivity and amount of tissue usually available

High infectious titre, no limitations in quantity	Low infectious titre expected, no limitations in quantity		Infectivity expected, limitations in quantity
Brain	Lymph nodes	Rectum	Ganglia
Spinal cord	Spleen	Skeletal muscle	Nerves
	Thymus	Tongue (muscular)	Nictating membrane
	Abomasum	Kidney	Tonsils
	Adipose tissue (mesentery)	Liver	Peyer's patches
	Caecum	Mammary gland	Adrenal gland
	Colon	Nasal mucosa	Bone marrow
	Duodenum	Pancreas	Eye (retina and optical nerve)
	Fore-stomach	Placenta	Milk cells
	Jejunum (other than Peyer's patches)	Salivary gland	White blood cells
	Ileum (other than Peyer's patches)	Blood: whole blood, plasma, red blood cells	
	Oesophagus	Cerebrospinal fluid	

These two aspects have direct consequences on the final testing protocol that could be applied.

4.4.1. Tissues with high infectious titre and no limitations in quantity

4.4.1.1. PrP^{Sc} detetction

IHC and WB detection should be systematically carried out as described in Section 4.3.2.

For WB, the last dilution displaying a positive result should be identified. The material used for WB should be identical (matched aliquot) to that used for evaluating the rapid tests.

4.4.1.2. Bioassay

An endpoint titration of posterior brainstem homogenate should be carried out by the inoculation of groups of six animals with dilutions up to 10^{-3}, and 12 animals with dilutions from 10^{-4} to 10^{-10}. The dilution series should be prepared using material that is identical (aliquot) to that used for evaluating the rapid tests.

The infectious titre in the brainstem of each animal should be established using the Spearman–Karber method. Data collected from animals inoculated with the posterior brainstem should be used to define a dose–response equation, as described in Arnold et al. (2009), allowing the establishment of the relative infectious titre of any other inoculum using the incubation period observed in mice inoculated with tissue homogenate.

In order to obtain information on the potential variation in the infectivity distribution in the brain of H-BSE and L-BSE-infected cattle, additional bioassays may be carried out. For the spinal cord, thalamus,

cerebral cortex and cerebellum, groups of six mice could be inoculated with a 10^{-2} dilution. This experiment could also allow the estimation of the final infectivity load potentially found in the whole brain of cattle infected with H-BSE and L-BSE.

4.4.1.3. Seeding activity

Posterior brainstem, spinal cord, thalamus, cerebral cortex and cerebellum 1/10 dilution series should be prepared (up to 10^{-15}) and tested, following the protocol described in Section 4.3.3. The material used should be identical to that used for bioassay. At least 20 negative controls (10 unseeded reactions and 10 reactions seeded with negative tissue) should be included in each amplification run.

4.4.1.4. Performance of rapid tests used in TSE surveillance in cattle

Rapid PrP^{Sc} detection assays, when applied to H-BSE and L-BSE detection, should be carried out using the brain material collected in the experimental H-BSE and L-BSE cases.

4.4.2. Tissues with low infectious titre expected and no limitations in quantity

4.4.2.1. PrP^{Sc} detection

For solid tissues, IHC and WB detection should be systematically carried out as described in Section 4.3.2.

For WB, the samples should initially be tested as a neat homogenate. If WB is positive, a dilution series should be prepared, and the last dilution displaying a positive result identified.

4.4.2.2. Bioassay

Tissue homogenates and body fluids should be inoculated in groups of 12 to 18 animals. Inoculation should be carried out using the same material (aliquot) as that used for WB. Attack rate and incubation period should be used to estimate the infectious titre using the dose–response function established using brain material. If no transmission is observed, the maximum infectious titre that could be present in the sample will be statistically estimated (see Section 3.1.2).

4.4.2.3. Seeding activity

A 1/10 dilution series will be prepared (up to 10^{-10}) and tested as described in Section 4.3.3. The material to be used should be identical to that used for the bioassay. At least 20 negative controls (10 unseeded reactions and 10 reactions seeded with negative tissue) should be included in each amplification run.

4.4.3. Tissues with infectivity expected and limitations in quantity

4.4.3.1. PrP^{Sc} detection

For solid tissues, IHC detection should be systematically carried out as described in Section 4.3.2.

Because of the limitations resulting from the volume of tissue homogenate, no WB should be performed in order to preserve the tissues and to prioritise bioassay.

4.4.3.2. Bioassay

Tissues homogenates and body fluids should be inoculated in groups of 12 to 18 animals. Attack rate and incubation period should be used to estimate the infectious titre using the dose–response function established using brain material. If no transmission is observed, the maximum infectious titre that could be present in the sample should be statistically estimated (see Section 3.1.2).

4.4.3.3. Seeding activity

A 1/10 dilution series should be prepared (up to 10^{-10}) and tested as described in Section 4.3.3. The material used should be identical to that used for the bioassay. At least 20 negative controls (10 unseeded reactions and 10 reactions seeded with negative tissue) should be included in each amplification run.

4.5. Estimation of analyses needed to implement the protocol

Tables 13–16 in Appendix B provide an estimate of the number of PrP^{Sc} detection analyses, bioassay (number of mice to be inoculated) and PMCA/QuIC reactions to be performed under different scenarios, and give details of the individual tissues to be tested.

The first scenario considers the tissues obtained through the EURL study, while the other three scenarios are based on the three tissue categories listed in Table 7, and would give an indication of the resources needed according to the three priority levels discussed in Section 4.1.2.

Table 9 below summarises the overall number of analyses needed.

Table 9: Estimation of the overall number of analyses needed **per inoculated cow** to implement the protocol under different scenarios (where appropriate, minimum and maximum number of analyses is reported). Details of the required analyses for single tissues are provided in Tables 13–16 in Appendix B

Scenarios	IHC	WB	Bioassay	Prion seeding activity reaction
Tissues obtained from the EURL study	19[(a)]	32–160	318–450	1 112
Priority 1 (cattle SRM list)	21	26–130	294–414	1 024
Priority 2 (non-SRM with low infectivity (or PrP^{Sc} presence) in ruminants and significant input into the food chain)	11	14–70	144–216	528
Priority 3 (non-SRM with low infectivity (or PrP^{Sc} presence) in ruminants and limited/no input into the food chain)	9	24–120	168–252	616

(a): IHC analyses have been already carried out in the framework of the EURL study.

4.6. Concluding remarks

- The application of the proposed protocol would provide elements allowing the assessment of the relative infectious titre, PrP^{Sc} accumulation and prion seeding activity in the tissues of cattle that developed H-BSE or L-BSE (using posterior brainstem as a reference).
- Tissues to be covered by further studies are categorised in three priorities, based on their inclusion in the cattle SRM list, on the presence of infectivity, or PrP^{Sc} presence, demonstrated in Atypical BSEs or other TSEs in ruminants, and on the importance in terms of input into the food chain in the EU.
- Applying the protocol only to the tissues obtained through the EURL study would provide information on some but not all the tissues from the cattle SRM list. It would also provide information on some additional tissues not included in the cattle SRM list, but relevant for the food chain.
- Material from other studies could be used to augment the range of SRM and non-SRM tissues available.

- There is no identified source able to provide all the samples necessary to assess infectivity in tissues belonging to the full cattle SRM list in H- and L-BSE-infected animals. Therefore, to complete this objective, new inoculation of cattle would have to be considered.
- In accordance with previous EFSA recommendations, through the implementation of the protocol information should also be obtained on the performance of currently validated rapid tests for TSE active surveillance in cattle/bioassay for detecting H-BSE and L-BSE agents.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

- Data relating to the prevalence and geographical distribution of Atypical BSE are incomplete.
- The recent cessation of the testing of healthy slaughtered cattle in some EU Member States will lead to a loss of capacity of the monitoring system to detect Atypical BSE cases.
- For transmission studies, i.c. challenge would be an appropriate proxy for studying the distribution of the agent if the origin of the disease was spontaneous, and originating in the brain, while oral challenge would be more appropriate if the origin of the disease was through ingestion of infected material.
- The current lack of information on the distribution of infectivity in tissues of Atypical BSE-infected cattle does not allow judgement of whether the current list of bovine SRM, set by EU legislation based on data relating to the pathogenesis and tissue distribution of C-BSE, is fit for the purpose of removing most of the Atypical BSE infectivity from bovine carcasses.
- Where data exist from both field cases and experimental animals (i.e. for L-BSE only), there is good agreement of the data with regard to abnormal PrP distribution. There are no data for field case H-BSE.
- Disease-related PrP has been reported consistently in CNS tissues, peripheral ganglia and nerves, muscles (predominantly muscle spindles), adrenal glands and retina for both H-BSE and L-BSE. All of these tissues are also positive in C-BSE.
- By contrast with C-BSE, at this stage no lymphoid tissues or gastrointestinal tissues from H-BSE- and L-BSE-affected animals have tested positive for PrP^{Sc} presence (IHC, WB) or infectivity (bioassay).
- The reference method for the estimation of prion infectious titre in tissues is endpoint dilution titration in animals. To achieve maximum sensitivity regarding Atypical H-BSE and L-BSE, this bioassay should ideally be done in mouse lines over-expressing bovine PrP^{C}. Several mouse lines over-expressing bovine PrP^{C} are available worldwide.
- *In vitro* amplification techniques can be used to determine whether a tissue contains any prion seeding activity. A correlation must be made between the sensitivity achieved by the cell-free assays and bioassays using reference material such as brain tissue from animals at the terminal stage of disease.
- The application of the proposed protocol would provide elements allowing the assessment of the relative infectious titre, PrP^{Sc} accumulation and prion seeding activity in the tissues of cattle that developed H-BSE or L-BSE (using posterior brainstem as a reference).
- Tissues to be covered by further studies are categorised in three priorities, based on their inclusion in the cattle SRM list, on the presence of infectivity, or PrP^{Sc} presence, demonstrated in Atypical BSEs or other TSEs in ruminants, and on the importance in terms of input into the food chain in the EU.

- Applying the protocol only to the tissues obtained through the EURL study would provide information on some but not all the tissues from the cattle SRM list. It would also provide information on some additional tissues not included in the cattle SRM list, but relevant for the food chain.
- Material from other studies could be used to augment the range of SRM and non-SRM tissues available.
- There is no identified source able to provide all the samples necessary to assess infectivity in tissues belonging to the full cattle SRM list in H- and L-BSE-infected animals. Therefore, to complete this objective, new inoculations of cattle would have to be considered.

RECOMMENDATIONS

- In accordance with former EFSA recommendations, through the implementation of the protocol information should also be obtained on the performance of currently validated rapid tests for TSE active surveillance in cattle/bioassay for detecting H-BSE and L-BSE agents.
- If new inoculation experiments are carried out in cattle with H-BSE and L-BSE, the following should be considered:
  - inoculation through both the i.c. and oral route (despite the potential length of the oral route experiment);
  - inclusion of C-BSE controls in the i.c. route experiment;
  - sequential time killing of animals;
  - collection of all tissues listed in Table 7.

DOCUMENTATION PROVIDED TO EFSA

1. Summary of the samples available and the tests already carried out by the EURL-TSE. Submitted by the European Commission as Annex 1 to the mandate.
2. Information on protocols and tests results provided by the EURL-TSE. Submitted by the European Commission as Annex 2 to the mandate.

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APPENDICES

Appendix A. Data from published studies on Atypical BSE and Classical BSE positivity

Table 10: Data available from published studies on H-BSE

Reference	**EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)**						**Buschmann et al. (2011a, b); Hoffman et al. (2007)**	**Okada et al. (2011, 2013)**	**Greenlee et al. (2012)**
TSE type	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE
Challenge status	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge
Animal ID	PG0384/10	PG1129/10	PG0373/10	PG0006/13	PG0782/10	PG0106/13	FLI-RA10, 13, 14, 15, 16	7749, 9458, 0728	
Breed	Aberdeen Angus X	Aberdeen Angus X	Aberdeen Angus X	Hereford X	Aberdeen Angus X	Hereford X	Holstein-Friesian	Holstein	E211K genotype
Sex	F	M	M	M	M	F	F	F(2), M(1)	F
Donor	French field case	French field case	French field case	sub-passage of 373/10	French field case	sub-passage of 782/10	German field case R152/04	Canadian H-BSE Charolais cross	2006 U.S. H-type BSE case associated with the E211K polymorphism
Age at challenge	11 months	10 months 2 weeks	10 months 3 weeks	7 months 1 week	11 months	7 months 1 week	6 months	3-4 months	2 months
Incubation period (days) - all animals left to develop to clinical end-point	559	582	526	505	649	525	365 early clinical, 419, 428, 475, 482	559.7 ± 47.2	288
Central nervous system									
brain	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB
spinal cord	IHC/WB	IHC	IHC		IHC		IHC/WB	IHC/WB	IHC
Peripheral nervous system									
Ganglia and plexi									
aorticorenal ganglion									
brachial plexus								IHC/WB	
caudal cervical vertebral ganglion									
caudal mesenteric ganglion									
coeliacomesenteric ganglion								IHC/WB	
cranial cervical ganglion								IHC/WB	
cranial mesenteric ganglion									
dorsal root ganglion								IHC/WB	
middle cervical ganglion									
nodose ganglion									
pelvic plexus									
stellate ganglion								IHC/WB	
thoracic vertebral ganglion									
trigeminal Ganglia	IHC	IHC	IHC		IHC			IHC/WB	IHC

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Okada et al. (2011, 2013)	Greenlee et al. (2012)
TSE type	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE
Nerves									
accessory nerve								IHC/WB	
facial nerve								IHC/WB	
hypoglossal nerve								IHC/WB	
median nerve								IHC/WB	
optic nerve								IHC/WB	IHC
phrenic nerve								IHC/WB	
radial nerve								IHC/WB	
sciatic nerve								IHC/WB	IHC
splanchnic nerve									
suprascapular nerve								IHC/WB	
tibial nerve								IHC/WB	
trigeminal nerve									IHC
vagus nerve								IHC/WB	
vagosympathetic trunk								IHC/WB	
peripheral nerves (unsp)									
Lymphoid tissue									
anterior cervical LN									
axillary LN								IHC/WB	
brachiocephalic LN								IHC/WB	
cervical LN								IHC/WB	
cervical Thymus								IHC/WB	
external iliac LN								IHC/WB	
GALT (various locations)									
hepatic LN								IHC/WB	
ileocaecal LN									
internal iliac LN								IHC/WB	
lateral retropharyngeal LN								IHC/WB	IHC
lymph node (unsp)									
medial retropharyngeal LN	IHC	IHC	IHC		IHC				IHC
mesenteric LN	IHC	IHC	IHC		IHC			IHC/WB	IHC
nictitating membrane									
palatine tonsil	IHC	IHC	IHC		IHC			IHC/WB	IHC
parotid LN								IHC/WB	
popliteal LN								IHC/WB	IHC
prefemoral LN									
prescapular LN									IHC
RAMALT									
splenic LN									
subiliac LN								IHC/WB	
submandibular LN								IHC/WB	
suprainguinal LN								IHC/WB	

Reference	**EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)**						**Buschmann et al. (2011a, b); Hoffman et al. (2007)**	**Okada et al. (2011, 2013)**	**Greenlee et al. (2012)**
TSE type	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE
spleen								IHC/WB	
Gastrointestinal tissues									
abomasum									IHC
adipose tissue (mesenteric)									
caecum									
colon									
colon (Peyer's patches)									
cranial oesophagus									IHC
distal ileum (Peyer's patches)	IHC	IHC	IHC		IHC				
duodenum									
ileocaecal plica (Peyer's patches)									
ileocaecal valve									
ileum (no Peyer's patches)									
ileum (unsp)									IHC
jejunum (no Peyer's patches)									
jejunum (Peyer's patches)									
jejunum (unsp)									
omasum									IHC
rectum (Peyer's patches)									
rectum									
reticulum									IHC
rumen									IHC
small intestine (unsp)									
large intestine (unsp)									
Muscles									
biceps									
deltoid									
diaphragm									IHC
ext abdominal oblique									
ext carpi radialis									
ext carpi ulnaris									
extraocular	IHC	IHC	IHC		IHC				
gastrocnemius									
gracilis									
heart									IHC
intercostal									
lat digital extensor									
longissimus dorsi									
masseter									IHC
medial gluteal	IHC[(a)]	IHC[(a)]	IHC[(a)]		IHC[(a)]				
pectoral									
peroneus									

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Okada et al. (2011, 2013)	Greenlee et al. (2012)
TSE type	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE	H-BSE
psoas									
semimembranosus									
semitendinosus	IHC[a]	IHC[a]	IHC[a]		IHC[a]				
tensor fasciae latae									
tongue									IHC
trapezius									
triceps	IHC[a]	IHC[a]	IHC		IHC[a]				
other muscles (unsp)									
Other tissues									
adrenals								IHC/WB	IHC
aorta									IHC
bladder									IHC
bone marrow									
eye							(retina)	(retina) IHC/WB	(retina) IHC
kidney									IHC
liver									IHC
lung									IHC
mammary gland									
nasal mucosa									IHC
oral mucosa									
parotid salivary gland									
skin									IHC
submandibular salivary gland									
thyroid									IHC
uterus									
Body fluids									
buffy coat (citrate)									
buffy coat (EDTA)									
cerebrospinal fluid									
milk									
plasma (citrate)									
plasma (EDTA)									
serum									
whole blood (citrate)									
whole blood (EDTA)									

Red	positive result with method indicated	GALT	gut-associated lymphoid tissue
Blue	negative result with method indicated	IHC	immunohistochemistry
Shaded grey	tissue available	LN	lymph node
unsp	unspecified	PMCA	protein misfolding cyclic amplification
(a)	negative but no muscle spindles in the sample	RAMALT	rectoanal mucosa-associated lymphoid tissue
		WB	Western blotting

Table 11: Data available from published studies on L-BSE

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Lombardi et al. (2008); Suardi et al. (2012)	Lombardi et al. (2008); Suardi et al. (2012)	Fukuda et al. (2009); Iwamaru et al. (2010)	Mazza et al. (2007); Suardi et al. (2012)	Suardi et al. (2012)	Casalone et al. (2004)
TSE type	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE
Challenge status	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	i.c. challenge	field case	field case	field case
Animal ID	PG1148/10	PG1345/10	PG0323/10	PG0101/13	PG1346/10	PG0107/13	FLI-RA01 to 06	254-F, 259-F, 261-F	816-Ab, 994-Ab, 995-Ab	528, 1061, 5566 (8515, 486 preclinical)	12966/07	126752/09	141387
Breed	Aberdeen Angus X	Aberdeen Angus X	Aberdeen Angus X	Hereford X	Aberdeen Angus X	Hereford X	Holstein-Friesian	Holstein-Friesian (3)	Alpine brown (3)	Holstein (5)	Piemontese (14y, 3rd BASE case)	Holstein-Freisian	Bruna alpina (2nd L-BSE field case)
Sex	F	F	M	M	M	F	F					F	
Donor	Italian field case (141387)	Italian field case (141387)	Italian field case (141387)	sub-passage of 323/10	Italian field case (141387)	sub-passage of 1346/10	German field case R172/02	BASE-affected Piemontese (code #1088)	BASE-affected Piemontese (code #1088)	aged Japanese Black cow JP24	NA	NA	
Age at challenge	10 months 1 week	10 months 2 weeks	10 months 1 week	7 months 1 week	10 months 2 weeks	7 months 1 week	6 months	4 months	4 months	2-3 months	NA	NA	
Incubation period (days) - all animals left to develop to clinical end-point	587	649	532	525	690	586	160 preclinical, 353, 417 early clinical, 466, 479, 489	461, 470, 480	525, 530, 551	486 ± 11			
Central nervous system													
brain	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB	IHC/WB/bioassay	IHC/WB	IHC/WB/bioassay		IHC/WB
spinal cord	IHC	IHC	IHC		IHC		IHC/WB	IHC/WB	IHC/WB	WB	IHC/WB		
Peripheral nervous system													
Ganglia and plexi													
aorticorenal ganglion													
brachial plexus										WB			
caudal cervical vertebral ganglion													
caudal mesenteric ganglion													
coeliacomesenteric ganglion													
cranial cervical ganglion													
cranial mesenteric ganglion										WB			
dorsal root ganglion													
middle cervical ganglion										WB			
nodose ganglion													
pelvic plexus													
stellate ganglion										WB			
thoracic vertebral ganglion													
trigeminal ganglia	IHC (inc)	IHC	IHC (inc)		IHC					WB			
Nerves													
accessory nerve										WB			
facial nerve										WB			
hypoglossal nerve										WB			

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Lombardi et al. (2008); Suardi et al. (2012)	Lombardi et al. (2008); Suardi et al. (2012)	Fukuda et al. (2009); Iwamaru et al. (2010)	Mazza et al. (2007); Suardi et al. (2012)	Suardi et al. (2012)	Casalone et al. (2004)
TSE type	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE
median nerve										WB			
optic nerve										WB	WB		
phrenic nerve										WB			
radial nerve										WB			
sciatic nerve										WB			
splanchnic nerve													
suprascapular nerve										WB			
tibial nerve										WB			
trigeminal nerve													
vagus nerve										WB			
vagosympathetic trunk										WB			
peripheral nerves (unsp)								IHC/WB	IHC/WB		IHC/WB		
Lymphoid tissue													
anterior cervical LN										WB			
axillary LN										WB			
brachiocephalic LN										WB			
cervical LN								IHC/WB	IHC/WB				
cervical Thymus								IHC/WB	IHC/WB				
external iliac LN										WB			
GALT (various locations)											IHC/WB		
hepatic LN										WB			
ileocaecal LN											IHC/WB		
internal iliac LN										WB			
lateral retropharyngeal LN										WB			
lymph node (unsp)									bioassay				
medial retropharyngeal LN	IHC	IHC	IHC		IHC								
mesenteric LN	IHC	IHC	IHC		IHC			IHC/WB	IHC/WB/bioassay	WB	IHC/WB		
nictitating membrane											IHC/WB		
palatine tonsil	IHC	IHC	IHC		IHC					WB			
parotid LN										WB			
popliteal LN										WB			
prefemoral LN													
prescapular LN													
RAMALT											IHC/WB		
splenic LN										WB			
subiliac LN										WB			
submandibular LN										WB			
suprainguinal LN										WB			
spleen								IHC/WB	IHC/WB	WB	IHC/WB/bioassay		
Gastrointestinal tissues													
abomasum													
adipose tissue (mesenteric)													

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Lombardi et al. (2008); Suardi et al. (2012)	Lombardi et al. (2008); Suardi et al. (2012)	Fukuda et al. (2009); Iwamaru et al. (2010)	Mazza et al. (2007); Suardi et al. (2012)	Suardi et al. (2012)	Casalone et al. (2004)
TSE type	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE
caecum													
colon													
colon (Peyer's patches)													
cranial oesophagus													
distal ileum (Peyer's patches)	IHC	IHC	IHC		IHC								
duodenum													
ileocaecal plica (Peyer's patches)													
ileocaecal valve											IHC/WB		
ileum (no Peyer's patches)													
ileum (unsp)											IHC/WB		
jejunum (no Peyer's patches)													
jejunum (Peyer's patches)													
jejunum (unsp)											IHC/WB		
omasum											IHC/WB		
rectum (Peyer's patches)													
rectum													
reticulum													
rumen											IHC/WB		
small intestine (unsp)													
large intestine (unsp)													
Muscles													
biceps												IHC	
deltoid									IHC				
diaphragm													
ext abdominal oblique												IHC	
ext carpi radialis												IHC	
ext carpi ulnaris												IHC	
extraocular	IHC	IHC	IHC		IHC								
gastrocnemius									IHC				
gracilis												IHC	
heart											IHC/WB		
intercostal											IHC/WB/bioassay	IHC	
lat digital extensor												IHC	
longissimus dorsi								IHC	IHC/bioassay			IHC	
masseter											IHC/WB		
medial gluteal	IHC[a]	IHC[a]	IHC[a]		IHC				IHC		bioassay		
pectoral									IHC			IHC	
peroneus												IHC	
psoas									IHC				
semimembranosus												IHC	
semitendinosus	IHC[a]	IHC[a]	IHC[a]		IHC[a]							IHC	
tensor fasciae latae												IHC	

Reference	EURL study (based on Konold et al. (2012) and other unpublished data provided by EURL in the framework of this mandate)						Buschmann et al. (2011a, b); Hoffman et al. (2007)	Lombardi et al. (2008); Suardi et al. (2012)	Lombardi et al. (2008); Suardi et al. (2012)	Fukuda et al. (2009); Iwamaru et al. (2010)	Mazza et al. (2007); Suardi et al. (2012)	Suardi et al. (2012)	Casalone et al. (2004)
TSE type	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE	L-BSE
tongue											IHC/WB		
trapezius												IHC	
triceps	IHC[(a)]	IHC[(a)]	IHC		IHC[(a)]			IHC/WB	IHC/WB			IHC	
other muscles (unsp)								IHC/WB	IHC/WB		IHC/WB		
Other tissues													
adrenals										WB			
aorta											IHC/WB		
bladder											IHC/WB		
bone marrow													
eye							(retina)						
kidney									bioassay		IHC/WB/bioassay		
liver								IHC/WB	IHC/WB		IHC/WB		
lung								IHC/WB	IHC/WB				
mammary gland											IHC/WB		
nasal mucosa											IHC/WB		
oral mucosa											IHC/WB		
parotid salivary gland													
skin													
submandibular salivary gland													
thyroid													
uterus											IHC/WB		
Body fluids													
buffy coat (citrate)													
buffy coat (EDTA)													
cerebrospinal fluid													
milk													
plasma (citrate)													
plasma (EDTA)													
serum													
whole blood (citrate)													
whole blood (EDTA)													

Red	positive result with method indicated	GALT	gut-associated lymphoid tissue
Blue	negative result with method indicated	IHC	immunohistochemistry
Shaded grey	tissue available	inc	inconclusive result
unsp	unspecified	LN	lymph node
(a)	negative but no muscle spindles in the sample	RAMALT	rectoanal mucosa-associated lymphoid tissue
		WB	Western blotting

Table 12: Data available from published studies on C-BSE

Reference	**Buschmann and Groschup (2005); Balkema-Buschmann et al. (2011a); Fast et al. (2013); Franz et al. (2012); Fukudaet al. (2012); Hoffmann et al. (2011); Iwata et al. (2006); Kaatz et al. (2012); Masujin et al. (2007); Okada et al. (2013); Stack et al. (2011); Terry et al. (2003); Wells et al. (2005)**
TSE type	C-BSE
Challenge status	experimental and field cases
Animal ID	various
Breed	various
Sex	various
Donor	various
Age at challenge	various
Incubation period (days) - all animals left to develop to clinical end-point	various
Central nervous system	
brain	IHC/WB/Bioassay
spinal cord	IHC/WB/Bioassay
Peripheral nervous system	
Ganglia and plexi	
aorticorenal ganglion	
brachial plexus	
caudal cervical vertebral ganglion	bioassay
caudal mesenteric ganglion	IHC/bioassay
coeliacomesenteric ganglion	IHC/bioassay/PMCA
cranial cervical ganglion	IHC/bioassay
cranial mesenteric ganglion	
dorsal root ganglion	WB/PMCA
middle cervical ganglion	
nodose ganglion	IHC/bioassay
pelvic plexus	
stellate ganglion	IHC/WB/bioassay/PMCA
thoracic vertebral ganglion	
trigeminal ganglia	IHC/bioassay
Nerves	
accessory nerve	
facial nerve	bioassay
hypoglossal nerve	
median nerve	
optic nerve	bioassay/PMCA
phrenic nerve	WB
radial nerve	WB/bioassay
sciatic nerve	WB/bioassay
splanchnic nerve	IHC/bioassay
suprascapular nerve	
tibial nerve	
trigeminal nerve	
vagus nerve	IHC/WB/bioassay
vagosympathetic trunk	
peripheral nerves (unsp)	
Lymphoid tissue	
anterior cervical LN	
axillary LN	WB
brachiocephalic LN	
cervical LN	WB
cervical Thymus	
external iliac LN	
GALT (various locations)	
hepatic LN	
ileocaecal LN	
internal iliac LN	WB
lateral retropharyngeal LN	PMCA
lymph node (unsp)	
medial retropharyngeal LN	
mesenteric LN	bioassay
nictitating membrane	
palatine tonsil	bioassay/PMCA
parotid LN	
popliteal LN	PMCA

Reference	Buschmann and Groschup (2005); Balkema-Buschmann et al. (2011a); Fast et al. (2013); Franz et al. (2012); Fukudaet al. (2012); Hoffmann et al. (2011); Iwata et al. (2006); Kaatz et al. (2012); Masujin et al. (2007); Okada et al. (2013); Stack et al. (2011); Terry et al. (2003); Wells et al. (2005)
TSE type	C-BSE
prefemoral LN	
prescapular LN	
RAMALT	
splenic LN	
subiliac LN	
submandibular LN	
suprainguinal LN	WB
spleen	bioassay/PMCA
Gastrointestinal tissues	
abomasum	PMCA
adipose tissue (mesenteric)	
caecum	
colon	
colon (Peyer's patches)	
cranial oesophagus	PMCA
distal ileum (Peyer's patches)	IHC/WB/bioassay/PMCA
duodenum	IHC/WB
ileocaecal plica (Peyer's patches)	
ileocaecal valve	IHC/bioassay
ileum (no Peyer's patches)	
ileum (unsp)	
jejunum (no Peyer's patches)	
jejunum (Peyer's patches)	IHC/WB/bioassay/PMCA
jejunum (unsp)	
omasum	
rectum (Peyer's patches)	
rectum	PMCA
reticulum	
rumen	PMCA
small intestine (unsp)	
large intestine (unsp)	
Muscles	
biceps	PMCA
deltoid	
diaphragm	
ext abdominal oblique	
ext carpi radialis	
ext carpi ulnaris	
extraocular	
gastrocnemius	
gracilis	
heart	bioassay
intercostal	IHC/WB
lat digital extensor	
longissimus dorsi	bioassay/PMCA
masseter	IHC/WB
medial gluteal	
pectoral	
peroneus	
psoas	IHC/WB/PMCA
semimembranosus	
semitendinosus	IHC/WB/ bioassay/PMCA
tensor fasciae latae	
tongue	bioassay/PMCA
trapezius	
triceps	IHC/WB
other muscles (unsp)	IHC/WB (quadriceps)
Other tissues	
adrenals	WB/bioassay/PMCA
aorta	
bladder	
bone marrow	bioassay/PMCA
eye	bioassay
kidney	
liver	

Reference	Buschmann and Groschup (2005); Balkema-Buschmann et al. (2011a); Fast et al. (2013); Franz et al. (2012); Fukudaet al. (2012); Hoffmann et al. (2011); Iwata et al. (2006); Kaatz et al. (2012); Masujin et al. (2007); Okada et al. (2013); Stack et al. (2011); Terry et al. (2003); Wells et al. (2005)
TSE type	C-BSE
lung	
mammary gland	
nasal mucosa	bioassay
oral mucosa	
parotid salivary gland	
skin	
submandibular salivary gland	
thyroid	
uterus	
Body fluids	
buffy coat (citrate)	
buffy coat (EDTA)	
cerebrospinal fluid	bioassay
milk	bioassay
plasma (citrate)	
plasma (EDTA)	
serum	
whole blood (citrate)	
whole blood (EDTA)	
Red	positive result with method indicated
Blue	negative result with method indicated
Shaded grey	tissue available
GALT	gut-associated lymphoid tissue
IHC	immunohistochemistry
LN	lymph node
PMCA	protein misfolding cyclic amplification
RAMALT	rectoanal mucosa-associated lymphoid tissue
WB	Western blotting
unsp	unspecified
(a)	negative but no muscle spindles in the sample

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Appendix B. Estimation of the number of analyses needed to implement the protocol

Table 13: Estimation of the number of analyses needed **per inoculated cow** to implement the protocol on tissues obtained from the EURL study (where appropriate, minimum and maximum number of analyses is reported)

Tissue	IHC[(a)]	WB	Bioassay	Prion seeding activity reaction
Brain (> 12 m): cerebellum	1	2–10	0–6	64
Brain (> 12 m): cortex	1	2–10	0–6	64
Brain (> 12 m): posterior brainstem	1	2–10	102	64
Brain (> 12 m): thalamus	1	2–10	0–6	64
Spinal cord	1	2–10	0–6	64
Trigeminal ganglia (> 12 m)	1	0	12–18	44
Peripheral nerves: sciatic	1	0	12–18	44
Lymph nodes	1	2–10	12–18	44
Tonsil	1	0	12–18	44
Spleen	1	2–10	12–18	44
Thymus	1	2–10	12–18	44
Ileum (Peyer's patches)	1	0	12–18	44
Jejunum (Peyer's patches)	1	0	12–18	44
Oesophagus	1	2–10	12–18	44
Skeletal muscle	1	2–10	12–18	44
Adrenal gland	1	0	12–18	44
Eyes (> 12 m)	1	2–10	12–18	44
Kidney	1	2–10	12–18	44
Salivary gland	1	2–10	12–18	44
Blood: plasma	NA	2–10	12–18	44
Blood: white blood cells	NA	0	12–18	44
Blood: whole blood	NA	2–10	12–18	44
Cerebrospinal fluid	NA	2–10	12–18	44
Total per inoculated cow	**19**	**32–160**	**318–450**	**1 112**

(a): IHC analyses have been already carried out in the framework of the EURL study.
NA: not applicable: analysis cannot be performed on this type of tissue.

Table 14: Estimation of the number of analyses needed **per inoculated cow** to implement the protocol on tissues included in the cattle SRM list (Priority 1) (where appropriate, minimum and maximum number of analyses is reported)

Tissue[(a)]	IHC	WB	Bioassay	Prion seeding activity reaction
Brain (> 12 m): cerebellum	1	2–10	0–6	64
Brain (> 12 m): cortex	1	2–10	0–6	64
Brain (> 12 m): posterior brainstem	1	2–10	102	64
Brain (> 12 m): thalamus	1	2–10	0–6	64
Spinal cord	1	2–10	0–6	64
Autonomic ganglia	1	0	12–18	44
Dorsal root ganglia (> 30 m)	1	0	12–18	44
Trigeminal ganglia (> 12 m)	1	0	12–18	44
Nictitating membrane	1	0	12–18	44
Tonsil	1	0	12–18	44
Adipose tissue (mesentery)	1	2–10	12–18	44
Caecum	1	2–10	12–18	44
Colon	1	2–10	12–18	44
Duodenum	1	2–10	12–18	44
Ileum (Peyer's patches	1	0	12–18	44
Ileum (other than Peyer's patches)	1	2–10	12–18	44
Jejunum (Peyer's patches)	1	0	12–18	44
Jejunum (other than Peyer's patches)	1	2–10	12–18	44
Rectum	1	2–10	12–18	44
Splanchnic nerves (mesentery)	1	0	12–18	44
Eyes (> 12 m)	1	2–10	12–18	44
Total per inoculated cow	**21**	**26–130**	**294–414**	**1 024**

(a): provided that an appropriate quantity of tissue is/remains available when the study is initiated

Table 15: Estimation of the number of analyses needed **per inoculated cow** to implement the protocol on tissues not included in the cattle SRM list, with low infectivity (or PrP^{Sc} presence) in ruminants and significant input into the food chain (Priority 2) (where appropriate, minimum and maximum number of analyses is reported)

Tissue[(a)]	IHC	WB	Bioassay	Prion seeding activity reaction
Peripheral nerves: radial	1	0	12–18	44
Peripheral nerves: sciatic	1	0	12–18	44
Peripheral nerves: vagus	1	0	12–18	44
Thymus	1	2–10	12–18	44
Abomasum	1	2–10	12–18	44
Fore-stomach	1	2–10	12–18	44
Skeletal muscle	1	2–10	12–18	44
Tongue (muscular)	1	2–10	12–18	44
Bone marrow	1	0	12–18	44
Kidney	1	2–10	12–18	44
Liver	1	2–10	12–18	44
Milk cells	NA	0	12–18	44
Total per inoculated cow	**11**	**14–70**	**144–216**	**528**

(a): provided that an appropriate quantity of tissue is/remains available when the study is initiated
NA: not applicable: analysis cannot be performed on this type of tissue.

Table 16: Estimation of the number of analyses needed **per inoculated cow** to implement the protocol on tissues not included in the cattle SRM list, with low infectivity (or PrP^{Sc} presence) in ruminants and limited/no input into the food chain (Priority 3) (where appropriate, minimum and maximum number of analyses is reported)

Tissue[(a)]	IHC	WB	Bioassay	Prion seeding activity reaction
Spleen	1	2–10	12–18	44
Lymph nodes	1	2–10	12–18	44
Oesophagus	1	2–10	12–18	44
Adrenal gland	1	0	12–18	44
Salivary gland	1	2–10	12–18	44
Mammary gland	1	2–10	12–18	44
Nasal mucosa	1	2–10	12–18	44
Pancreas	1	2–10	12–18	44
Placenta	1	2–10	12–18	44
Blood: plasma	NA	2–10	12–18	44
Blood: red blood cells	NA	2–10	12–18	44
Blood: white blood cells	NA	0	12–18	44
Blood: whole blood	NA	2–10	12–18	44
Cerebrospinal fluid	NA	2–10	12–18	44
Total per inoculated cow	**9**	**24–120**	**168–252**	**616**

(a): provided that an appropriate quantity of tissue is/remains available when the study is initiated.
NA: not applicable: analysis cannot be performed on this type of tissue.