

Protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants¹

Scientific Opinion of the Panel on Biological Hazards

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SUMMARY

Annex X to Regulation (EC) No 999/2001 lays down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies and lists the approved rapid tests which may be used within the framework of the EU monitoring programmes. The approval of these tests was based on SSC and EFSA evaluation protocols and its recommendations on the suitability or otherwise of the evaluated tests for inclusion in the EU programme for TSE monitoring.

The EC will now launch a new open call for expressions of interest, for rapid tests for use in the framework of TSE monitoring. This call is intended to cover tests for TSE detection *ante-* and *post-mortem* in cattle and sheep and goats. Evaluation of these tests is based on a protocol developed by TSE testing experts and covers different steps including a pre-assessment, an assessment of the application dossier, a laboratory evaluation, approval of the package insert and a field trial. EFSA was asked by the EC to revise and update the three current protocols for the evaluation of TSE tests in ruminants taking into account the experience gained in past evaluation rounds.

This opinion reports on the revised protocol for the evaluation of *post mortem* TSE tests in small ruminants.

In 2003 the European Commission (EC) (DG SANCO and DG JRC and its IRMM) and EFSA, started evaluation of rapid tests for TSE epidemio-surveillance in small ruminants. During the previous evaluation process, differences were observed between tests in terms of analytical sensitivity. However,

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the significance of such differences both in term of field diagnostic sensitivity and biological relevance could not be scientifically assessed at the time of evaluation. Moreover, following the implementation of active surveillance programs in the EU using tests that were validated, a new type of TSE (atypical scrapie cases/NOR98) not previously recognized in the EU, was detected in small ruminants. Currently atypical/Nor98 has been detected in a large number of European countries and approximately constitutes 80% of test positive cases identified in EU. Data collected in this EU active surveillance program clearly indicate that all the validated tests do not perform equally toward atypical cases and that difference in performance result in under- or non recognition of various types of scrapie.

The EFSA Scientific Panel on Biological Hazards (BIOHAZ) has agreed on a revised evaluation protocol which takes into account the experience gained in past evaluation rounds and knowledge accumulated from the active surveillance program. New tests have to successfully pass all stages of the evaluation process. Progress to the next stage requires successful completion of the previous stage and therefore the process can be suspended at any stage of the evaluation. This protocol ensures that newly approved tests will not be inferior to previously approved BSE *post mortem* screening tests. In addition to previous evaluation criteria, the revised protocol considers each test's performance with respect to (i) detection of classical scrapie, atypical scrapie and BSE in sheep and (ii) detection of preclinical cases and (iii) limitations posed by analytical sensitivity in comparison with bioassay. The criteria in this revised protocol introduce more comprehensive and higher standards than have previously been approved for validation of small ruminant *post mortem* TSE tests for classical scrapie and BSE as well as for atypical scrapie. Considering data available about abnormal PrP distribution in the three recognized small ruminants TSE forms (BSE, classical scrapie and atypical scrapie) the use of brainstem appears to be the best compromise for detection of all TSE agents in small ruminants. In consequence, officially confirmed (by CRL and NRL) positive/negative brainstem will be used for the evaluation of tests.

The BIOHAZ panel recommends that tests already approved for the detection of TSE in small ruminants should be required to participate in the new evaluation in order to confirm their robustness and their ability to fulfil the additional performance requirements (*e.g.* atypical cases and analytical sensitivity). This re-iterates a recommendation of their recent Opinion on the EU TSE Community Reference Laboratory report on batch testing of TSE rapid tests: sample selection and test sensitivity issues². It is further recommended that tests that are not able to meet requirements for detection of all types of TSE (classical scrapie, BSE and atypical scrapie) not be considered for testing small ruminants in the field. Tests that fail to meet a requirement in respect of a particular tissue type (lymphoid/CNS) should not be recommended for application on that tissue. Finally, taking into account the experience gained in the TSE test batch testing protocol and because knowledge in the TSE field is rapidly evolving, the BIOHAZ panel recommends that a system of periodic re-assessment of test approval based on both test field performance and evolving EU policy objectives should be considered by the Risk Managers.

Key words: TSE, Transmissible Spongiform Encephalopathies, small ruminants, protocol, *post mortem* rapid tests.

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BACKGROUND

Annex X to Regulation (EC) No 999/2001 lays down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies and lists the approved rapid tests which may be used within the framework of the EU monitoring programmes. Only post-mortem tests have been approved for this purpose so far. The approval of these rapid post mortem tests was based on EFSA evaluation protocols and its recommendations on the suitability or otherwise of the evaluated tests for inclusion in the EU programme for TSE monitoring.

According to regulation 999/2001, CNS samples of small ruminants in the European Union have to be tested for the presence of transmissible spongiform encephalopathy (TSE). Eight rapid tests (Beckman Coulter Inpro CDI kit, Enfer TSE kit Version2, BioRad TeSeE, BioRad TeSeE sheep/goat test, Idexx Herd-Check BSE-Scrapie Antigen test kit EIA, Pourquiers LIA Scrapie, Prionics-Check LIA Small Ruminants and Prionics-Check Western Blot Small Ruminant test) are approved for the *post mortem* diagnosis of TSE in Small Ruminants (SSC, 2002a; EFSA 2005a; EFSA 2005b). Evaluation of these tests was coordinated and supervised by staff of The Institute for Reference Materials and Measurements (IRMM) of the Directorate General Joint Research Centre (DG-JRC) acting on behalf of Directorate General for Health and Consumer Protection (DG SANCO). Reports compiled and prepared by the IRMM were evaluated by an independent group of experts on TSE testing of the EFSA (EFSA TSE Testing Expert Group).

MANDATE

In the second half of 2007, the EC plans launching a new open call for expression of interest for rapid test for use in the framework of TSE monitoring. This call, whose general conditions will be set up by the EC and published in the Official Journal of the European Union (C series), is intended to cover tests for TSE detection *ante-* and *post-mortem* in cattle and sheep and goats. In order to achieve this, and in preparation, there is a need to update current TSE test evaluation protocols. These new protocols will be the basis for any future evaluation round of rapid *ante-* and *post mortem* tests.

The EFSA is requested to update the current protocols for the laboratory evaluation and field trial, taking into account experience gained in past evaluation rounds.

These protocols include:

- Scientific Report of the European Food Safety Authority on the Design of a Field Trial Protocol for the Evaluation of BSE Tests for Live Cattle adopted on 1 July 2004;
- Scientific Report of the European Food Safety Authority on the Design of a Field Trial Protocol for the Evaluation of New Rapid BSE post mortem Tests adopted on 5 April 2004;
- Opinion on a programme for the evaluation of rapid post mortem tests to detect TSE in small ruminants adopted by the Scientific Steering Committee at its meeting of 7-8 November 2002.

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1. DEFINITIONS

Sliced material

Tissue which is cut into pieces with a blade.

Macerates

The dictionary definition of macerate (Biology-online.org) is “to soften by steeping or soaking in liquid”. In this document the word macerate is used to describe all sample preparation methods where the tissue is prepared by adding liquid and allowing it to soak, pass through a sieve or otherwise dividing it into coarse pieces. The process is recognized to be gentle and suitable for preparing CNS prior to testing in TSE rapid tests.

Homogenates

The dictionary definition of homogenate (Biology-online.org) is chaotic slurry of tissues and cells which results when cell tissue structure has been disrupted by mechanical (as opposed to chemical) means, often by mincing or grinding. Homogenates may be prepared in a number of ways, but generally involve mechanical disruption of tissue using an automated instrument. The term homogenisation can prove confusing as not all homogenates have the same properties and attention must be given to the preparation method. If high speeds and shearing forces are used (for example by using an Ultra-Turrax) giving fine homogenates of TSE-positive brain material of a small particle size, this can have an adverse effect and even in render positive samples non-detectable by some tests. Homogenates prepared in such a manner are not permitted. However, more gentle methods, such as a low speed hand-held blender with metal or plastic blades, to prepare coarser samples, merely disrupts tissue and is a reliable preparation method for TSE samples giving samples with similar properties to a macerate (see annex 2 for CRL method).

Brain stem

The term brain stem is used in this opinion to refer to that part of the brain that is usually removed via the foramen magnum for subsequent rapid testing, and generally comprises the anatomical regions of the medulla oblongata, pons and cerebellar peduncles, with the exception of the obex which will usually have been removed for primary testing and confirmation.

2. PURPOSE OF THE EU WIDE SURVEILLANCE PROGRAMME

Active surveillance in the EU has been conducted as from January 2002 using BSE *post mortem* tests and in 2003 a first evaluation was made of the results. Concerns were expressed related to the sensitivity of these tests used for the TSE detection in sheep and goat, as these tests were validated only for BSE detection in cattle. A strong recommendation was formulated to approve tests in particular for TSE detection in small ruminants, to be used in a TSE surveillance programme to provide reliable data on the occurrence of TSE in small ruminants under natural conditions which takes into account factors such as geographical area, breed, age and genotype (EFSA, 2003).

Between 2003 and 2005 the EU (EFSA, in cooperation with JRC/IRMM) evaluated rapid tests for TSE epidemio-surveillance in small ruminants (EFSA, 2005a; EFSA, 2005b). The evaluation process was conducted following recommendations from scientific experts, reflected knowledge in the TSE field at the time of evaluation and resulted in approval of some of the proposed tests.

The sole objective of the current EU testing programme is epidemio-surveillance. However, it may be possible to consider other uses in the future, such as certification of (TSE-free) flocks.

3. PURPOSE OF THE REVIEW OF THE PROTOCOLS FOR TSE TEST EVALUATION

The purpose of the review of the protocol for TSE test evaluation in SMRU is to define the evaluation criteria to be used, taking account of:

- a. The detection of new TSE agents during on-going surveillance;
- b. The acquired knowledge during past evaluations;
- c. The necessity for assuring continuity with previous evaluation;
- d. The use of an approach that remains practical.

It should be applied where manufacturers have a test ready for use under field conditions, together with properly documented validation and quality data to support an application. This protocol is not appropriate for tests still undergoing development or those intended solely as research tools.

4. THE EVALUATION

Evaluation of TSE tests submitted following a call for expression of interest launched by the EC, comprises a series of steps. A test may be excluded from further assessment at any stage:

- a. The assessment of the submitted dossier by the EFSA TSE Testing Expert Group;
- b. A pre-evaluation assessment by the EFSA TSE Testing Expert Group;
- c. A laboratory evaluation supported by the preparation of a report, supervised by staff of the IRMM. IRMM is also responsible for the sample distribution, data collection and analysis, and reporting.
- d. The evaluation of the IRMM report and of the package insert for the test kit, developed prior to initial approval, carried out by EFSA TSE Testing Expert Group.

- e. For final approval an alternative approach to a field trial supported by the preparation of a report, supervised by the EU TSE CRL also responsible for the evaluation of the data. The EU TSE CRL is also responsible for the data collection and analysis, and reporting.

4.1. Assessment of submitted dossier

The manufacturer must provide information pertaining to all points listed in the call as part of their submission-dossier and supporting data.

- a. This should include results from testing a panel of at least 10 positive and 50 negative samples of each tissue type for which a claim is made (CNS/LRS). The samples should have also been tested using either an EU approved rapid test or OIE confirmatory method to define the provenance of the particular sample. The manufacturer should provide details about the origin of the samples (age and species of animals, type of tissue, age and storage conditions for sample, method used to prepare samples if macerates are used).
- b. Details of the method used to test samples and confirm status, where and when this testing was done and the results obtained.
- c. Any additional information demanded by the EC on test set up and performance

4.2. Pre-evaluation assessment

An initial assessment of each applicant will be performed before the formal evaluation begins. This evaluation will involve:

- a. Examination of the submitted dossier – see 4.1 above and conditions in the EC Call for expression of Interest
- b. A visit to the manufacturer's premises to see the test being performed, to inspect the kit production and quality control facilities and to clarify any issues arising from examination of the submitted dossier (IRMM in collaboration with CRL).
- c. During the visit the manufacturer will be required to test a typical panel of 20 proficiency test samples as issued by the TSE Community Reference Laboratory (CRL). This set will include a panel of dilution series to determine the analytical sensitivity of the assay on the basis of a selection of samples as specified in 8.3. This will allow the inspectors to make a preliminary assessment of the method, and compare performance with manufacturers' claims.
- d. The dilution series will be made from macerates of classical scrapie positive brain stem at clinical stage diluted with macerates of negative brain material. Tests should be able to detect at least 5% positive tissue in negative tissue. Equivalent samples will have been subjected to prior testing with a test having shown high analytical sensitivity performance during previous evaluations (EFSA, 2005a; EFSA, 2005b). For all positive samples, a confirmatory WB aiming at profile identification will have been carried out (using 0.5 grams of tissue and an anti-PrP antibody with at least equivalent sensitivity to Sha31 mAb as anti-PrP antibody).

All these data and materials will be treated confidentially by European Commission Services and will not be made available to third parties. They must be delivered before the actual evaluation exercise starts.

Closure of the pre-evaluation, and entry into full evaluation, requires a satisfactory assessment of the submitted dossier, a satisfactory report on the visit to the manufacturer's laboratory, and 100% accuracy on testing of the proficiency test panel and if the limit of detection (*i.e.* detection limit as determined by bioassay) for the selected samples provided is better than, similar to or no more than 2 logs poorer than the most sensitive (as specified in chapter 8.5.2.4.).

4.3. Full laboratory evaluation criteria

During the full evaluation, the submitted tests will be evaluated for one or more tissues (CNS and/or lymph nodes) depending on the requirements of the manufacturer. It is envisaged that successful tests will be approved for testing if:

- The particular tissues tested successfully in the evaluation;
- Only in accordance with the test parameters and test components specified in the evaluation *i.e.* the protocol used during the evaluation.

4.4. Field trial

Although a field trial component was included in previous evaluations of tests for BSE in bovines, such a stage was not applied to tests for small ruminants. The comprehensive nature of this evaluation, in terms of numbers of samples tested, and the various matrices supplied coupled with the current reduced scale of surveillance in small ruminants means that it is still not considered practical or proportionate to require a field trial stage. Nevertheless, in view of experiences with current tests and problems encountered on introduction into surveillance programmes, an alternative approach is to be applied.

Initial approval will be subject to completion of a satisfactory evaluation of raw data from a minimum of two testing laboratories into which the test has been introduced, totalling 10,000 negative samples. Following evaluation of the data, by the EU TSE CRL, provided that no evidence of problems with respect to performance are detected, the approval process will be validated by the EFSA TSE testing expert group.

5. OBJECTIVES OF THE EVALUATION PROTOCOL

Based on experience gained from earlier evaluations and field testing programs across EU, evaluation of the tests performance must concentrate on the estimation of:

- a. Diagnostic sensitivity
- b. Diagnostic specificity
- c. Repeatability
- d. Capacity to detect preclinical cases (classical scrapie).
- e. Capacity to detect cases linked to all different type of TSE agents (BSE, classical scrapie and atypical scrapie)

- f. Detection limit of each diagnostic test (analytical sensitivity/bioassay which is considered as a gold standard).

Although it is recognized that the tests are to be used for epidemio-surveillance, when used the detection limit is considered to be a critical point for the key issue of public health (particularly in the case of BSE) and for the capacity of a test to detect scrapie in small ruminants at early stages of incubation.

The proposed evaluation protocol tries to take into account all these aspects within the limits of:

- Actual availability of biological material
- Technical and calendar constraints
- In addition, concerns expressed in a recent opinion of EFSA (EFSA, 2005c) about atypical cases and active surveillance programs and a recent publication (Noremark and Hopp, 2006), called for a revisiting and re-evaluation of the already approved tests (annex 1 for supporting comments).

6. TSE AGENTS PATHOGENESIS AND CONSEQUENCES ON TARGET TISSUE CHOICE

Field TSE surveillance programmes aim to detect efficiently the different forms of TSE in small ruminants at preclinical and clinical stages. Apart from the direct performance of the test in terms of sensitivity and specificity, the ability to determine accurately the infectious status of an individual depends on the choice of appropriate tissues for testing. Pertinent choice relies on current knowledge concerning pathogenesis of different TSE agent in small ruminants.

6.1. Classical scrapie and BSE

6.1.1. PrP^{Sc} dissemination pathway in organism

During natural infection by TSE, transmission is generally considered to occur via the gastrointestinal route. After exposure, in sheep of susceptible genotype (other than ARR heterozygotes) and goats, PrP^{Sc} accumulation is usually, but not invariably, first detected in the in Peyer's Patched and lymph nodes associated with the digestive tract (Andreoletti *et al.*, 2000; Heggebo *et al.*, 2002), before extending progressively and accumulating in all other secondary lymphoid formations. Lympho-invasion is usually completed within the first half of incubation period, during which the agent is largely disseminated in the body but not detectable in CNS (Andreoletti *et al.*, 2000).

The lymphoid organs are richly innervated by sympathetic fibres (Felten and Felten, 1988), and it is hypothesized that these fibres are invaded through contact with infected lymph organs (Cullen *et al.*, 1984). The network of the gastrointestinal autonomic nervous system contains innumerable amyelinic nerve fibres, some of which innervate the superficial zone of the lymph follicles (Collis and Kimberlin, 1983). Currently the most widely accepted hypothesis explaining the penetration in the nerve compartment is that there is direct invasion of these fibres from the germinal centres. This hypothesis is supported by the early presence in sheep with scrapie (Heggebo *et al.*, 2003; Andreoletti *et al.*, 2000) of PrP^{Sc} in the neurones of the myenteric plexuses, located immediately next to the Peyer's patches.

Once in the autonomic nervous system, the pathogenic protein seems to progress to the central nervous system (CNS) simultaneously along two routes:

- The sympathetic nerve fibres (splanchnic nerves) which innervate the digestive organs and their associated lymph nodes. The entry of the agent by the intermediolateral column in the spinal cord in sheep naturally infected with scrapie confirms the involvement of these fibres in the dissemination of the agent towards the CNS (Andreoletti *et al.*, 2000).
- the parasympathetic fibres and notably those of the vagus nerve. The involvement of this route is indicated by the entry and early replication of PrP^{Sc} in the dorsal nucleus of the vagus nerve in sheep with scrapie (Cooley *et al.*, 2001).

CNS involvement usually takes place in the second half of the incubation period. This two-pronged dissemination is seen in various mammals, such as mouse (Maignien *et al.*, 1999), hamster (Beekes *et al.*, 1998; McBride *et al.*, 2001), sheep (Heggebo *et al.*, 2002; Andreoletti *et al.*, 2000) and large wild ruminants (cervids) (Sigurdson *et al.*, 2001). In sheep experimentally exposed to BSE agent through the oral route a similar dissemination scheme has been described, with an initial entry through the gut associated lymphoid tissue (GALT) and secondary dissemination to the CNS via the autonomic nervous system. (Jeffrey *et al.*, 2002; Bellworthy *et al.*, 2005).

6.1.2. Host and environment linked variations

In sheep and goats exposed to natural infection pressure, infection usually takes place very early in life (around birth). In adult animals, introduced into infected flocks, oral infection seems to occur, but with lower efficiency. Kinetics of dissemination dynamics seems also to depend on (1) TSE agent strains, (2) genotype of the host, and (3) exposure level.

Moreover in infected sheep that are heterozygous for the ARR allele (very low incidence of disease in these genotypes), involvement of lymphoid tissues seems to be extremely infrequent, and PrP^{Sc} can be detected in CNS while no PrP^{Sc} can be observed in lymphoid organs (Van Keulen, 1995; Andreoletti *et al.*, 2002). Similar findings have been reported in naturally infected animals harbouring fully susceptible genotype, indicating some variability into the general dissemination scheme (Jeffrey *et al.*, 2002).

6.2. Atypical scrapie

Given the current state of knowledge, it is not possible to confirm that the source and routes of infection, and subsequent pathogenesis, are similar in atypical and classical scrapie. In atypical scrapie cases, several other elements are relevant to diagnosis:

1. PrP^{Sc} accumulation is usually but not always, more intense in the cerebral cortex and/or cerebellum than in other brain area. However high inter-individual variation in abnormal PrP distribution was observed in brain from atypical cases, which in a proportion of cases results to an absence of PrP^{Sc} detection in cerebellum (Seuberlich *et al.*, 2007; Benestadt *et al.*, 2003).
2. There are currently no data to suggest that there is peripheral accumulation of PrP^{Sc} in affected small ruminants
3. High infectious titers (in transgenic ovine-mouse model) seem to be involved even when PrP^{Sc} is poorly detectable.

6.3. Factors influencing the final choice of tissue for diagnosis.

The sole objective of the current EU testing programme is epidemio-surveillance. However, it may be possible to consider other uses in the future, such as their use for certification of (TSE-free) flocks.

6.3.1. CNS / lymphoid tissue testing

In the context of classical scrapie and BSE in small ruminants, secondary lymphoid tissue, because of its early involvement in pathogenesis, may enable earlier detection of infected animals. Mesenteric or retropharyngeal lymph nodes, tonsil or spleen are roughly equivalent as potential target sites, with slight difference in terms of timing of PrP^{Sc} accumulation and detection. However, secondary lymphoid organs cannot be considered on their own as appropriate samples for individual diagnosis or epidemio-surveillance program, because of (1) poor involvement of lymphoid system in sheep of particular genotypes (heterozygote ARR sheep in particular), and (2) the inappropriateness of lymphoid tissue for the detection of atypical cases.

The CNS is involved in later stages of the incubation period (second half), which means that a negative result cannot prove that the tested animal is uninfected. The power of active surveillance programmes is therefore impaired by the inability to detect a proportion of incubating animals by testing brainstem only.

Clearly, the testing of lymphoid tissue or CNS, on its own, cannot be considered reliable for efficient individual diagnosis in small ruminants, and even in combination cannot guarantee a negative infectious status. The testing of both lymphoid tissue and CNS would obviously improve diagnostic sensitivity over and above the testing of CNS alone, but would introduce additional practical considerations.

6.3.2. CNS sample to be tested

Despite the variations in PrP^{Sc} distribution seen in both classical and atypical scrapie, scientific arguments and experience gathered during previous evaluation rounds and through the 2001-2007 active EU surveillance program, support the use of brainstem as the best compromise for detection all the TSE agents in sheep.

We therefore propose the use of officially qualified positive/negative brainstem (by OIE reference method and /or confirmatory test used in NRL) for the evaluation of tests.

Despite the fact that tests will be evaluated specifically (and so approved) for the testing of brain stem, it is recognized that field positive results obtained through the use of such tests on other parts of the brain are still interpretable. In the case of atypical scrapie in particular it is expected that other parts of the brain, such as cerebellum and frontal cortex, may provide improved test signals which could assist in the resolution of inconclusive or high negative readings at the obex. Negative results that would be obtained in other parts (than brain stem) of the brain only cannot be interpreted.

Taking into account past experience and currently available data the expert group would additionally recommend that in order to facilitate confirmation of atypical scrapie, cerebellum samples should also be collected.

7. SAMPLE SET AND PREPARATION

Referring to annex 1 of the SSC opinion of 22 February (SSC, 2002b) sensitivity and specificity of a diagnostic test should ideally be determined in comparison to a given ‘gold standard’. Such a ‘gold standard’ (bioassay) will not be available for each sample included in the evaluation process for the obvious reasons of cost and time. However for the critical aspect of sensitivity evaluation of each test, such gold standard reference will be included.

7.1. Numbers of samples to be tested

The following table summarises the type and numbers of samples for the evaluation. The testing of lymphoid tissue will be optional.

Table 1. Type and numbers of samples that will be needed to be evaluated during the laboratory evaluation.

Brain stem samples and/or lymph node*	Slices (sheep and goats)	Macerates (sheep and goats)	Autolysed samples	Total
Classical scrapie Positives	200	200	50 (or as many as possible)	450
Negatives	1000	200	50	1250

* The testing of lymphoid tissue will be optional and at the discretion of the company. In that case the same number of samples to be tested apply.

7.1.1. Positive samples

The OIE (Office International des Epizooties, Paris, France) recommends a sample size for the evaluation of diagnostic tests of 250 positives. This allows estimation of sensitivity of >99.5 % assuming the test screens a population with at least 1000 positive animals.

Because of possible modifications of the tissue linked to sample condition, and the particular conditions in which samples are collected in the field (rendering plant), 50 autolyzed samples from positive cases should also be evaluated. Autolysed field samples are to be preferred to samples autolysed under controlled conditions in a laboratory because of the potential differences in the properties of the resulting samples. Samples will be located and collected to include a range of genotypes and geographical origin. All sample material will be prepared and dispatched by the IRMM.

7.1.1.1. Clinical scrapie cases.

Brainstem and lymph nodes will be collected from naturally infected sheep or goats that showed clinical signs of TSE. These samples may originate from several EU member states. In each case the disease status of the animal should have been confirmed by (1) an OIE approved diagnostic method, usually histopathology, immunohistochemistry or OIE SAF Immunoblot or confirmatory WB and (2) prior to sample preparation, a previously approved rapid test with highest sensitivity (EFSA, 2005a; EFSA, 2005b). The PrP genotype of all animals will be determined. Samples will be frozen after collection and stored after preparation at -70°C.

7.1.1.2. Clinical sheep BSE cases.

CNS and lymphoid samples from experimentally infected sheep, of ARQ/ARQ and ARR/ARR genotype should be tested. In each case the disease status of the animal should have been confirmed by (1) an OIE approved diagnostic method (usually histopathology, immunohistochemistry or OIE SAF Immunoblot) or confirmatory WB and (2) prior to sample preparation, a previously approved rapid test with highest sensitivity (EFSA, 2005a; EFSA, 2005b).

7.1.1.3. Preclinical scrapie cases.

These samples will come from studies already initiated in naturally exposed research flocks where the prevalence in particular genotypes of sheep are extremely predictable, enabling certainty of status in the preclinical stage despite the fact that CNS testing may be negative at the time of collection. (groups of 5 to 10 infected animals killed at 7, 10 and 13 months plus 5 controls per group). In each case the disease status of the animal should have been confirmed by (1) an OIE approved diagnostic method (usually histopathology, immunohistochemistry or OIE SAF Immunoblot) or confirmatory WB and (2) prior to sample preparation, a previously approved rapid test with highest sensitivity (EFSA, 2005a; EFSA, 2005b).

7.1.1.4. Atypical scrapie cases.

Definition of atypical TSEs case in small ruminants as given in the EFSA opinion and its annex 1 (EFSA, 2005c).

Atypical TSEs in small ruminants

In 1998, the molecular and histopathological spectrum of TSEs in sheep was extended by the discovery in Norway of an experimentally-transmissible, PrP-related, neurological disease of sheep that was distinguishable from classical scrapie and was therefore considered to be an “atypical” form of scrapie. These Nor98 cases, the prototypes of “atypical” TSE, have little or no vacuolation or abnormal PrP at the obex, but in most cases exhibit an intense cerebellar PrP^{Sc} deposition/accumulation characterised at a molecular level by a smaller and less stable protease-resistant core of PrP^{Sc}. Nor98 and other “atypical” cases subsequently identified are more often but not uniquely, found in animals carrying alleles not usually associated with classical scrapie. For Nor98, this genotype correlation has been further refined recently to implicate another dimorphic codon in the PrP open reading frame, L141F. Other “atypical” TSE phenotypes, including those that are similar to or the same as Nor98 have now been published or in press/submitted from France, Germany, Sweden, Ireland, Portugal, Belgium and the UK.

Minimum numbers would include 10 (brain sample) which are all genotyped. All samples were primarily identified in the field using an approved rapid test and confirmed by NRL. Following preparation, IRMM will confirm samples status using TSE western blot (deposit per lane 15 mg equivalent tissue SHa 31 according to the French NRL protocol).

7.1.2. Negative samples

A lack in specificity in screening tests could lead to the detection of higher numbers of false positives causing undesired secondary effects. A panel of 1200 negative cases (1000 slices – 200 additional for

macerates evaluation) will be included in the evaluation process. According to OIE a 100% success in testing 1000 samples leads to a specificity of 99, 8% within a 95% confidence range

Samples will be collected from apparently healthy small ruminants. Ages of animals (minimal three years) will be noted and genotype for sheep at codons 136-141-154 and 171 of the PRP gene and for goats at codons 142-154-211 and 222 will be established.

By preference, complete sample sets from the same animal should be provided including material from CNS and lymph nodes. Each sample will be tested/screened by 2 approved small ruminant tests with the highest sensitivity. In the case of positive results (from the sample set collected as negatives) a confirmatory WB will be carried out and the sample will be discarded from the evaluation.

7.2. Difficulties and caveats in preparing sampling material

The tissue preparation procedure itself has a pivotal impact on the stability of PrP^{Sc} aggregates and it is assumed that repeated freezing and thawing has an effect on the aggregates. Therefore, these influences need consideration when trying to determine the true performance of a test (see Annex 3).

Because of these issues between procedures, it was not possible to consider the use of standardized sample panels for the 2001 evaluation of the different tests. The evaluation process was carried out using frozen non-homogenized tissues. Sub-sampling of tissues pieces was done according to an established rigorous permutation scheme. However this approach did not allow a direct comparison of the tests performance on equivalent material with low PrP concentration. This approach allowed evaluations of populations but not of individual samples.

Moreover, in the context of a very limited number of samples (in atypical preclinical cases) it was considered that evaluation may require to be performed using tissues macerates as this appeared to be the only way to ensure unambiguous comparison of test performance. Similarly, the most recent phase 2 evaluations (field trials) of tests for BSE *post mortem* tests was carried out using macerates (EFSA, 2004).

- Several parameters in the preparation of samples remain partially unknown, these include:
 - Equivalence between tissue slice/macerates
 - long term stability of PrP^{Sc} in macerates (which could impact assay performance to different degrees)
- In this context EFSA TSE Testing Expert Group considers that:
 - assuring continuity in the evaluation process between the current proposal and previous evaluations is needed
 - a comparison of the macerates/slice approaches is required in order to allow the use of macerate in future evaluation processes.
 - using macerates in some aspect of the evaluation (dilution sensitivity – atypical cases evaluation) is both inevitable and appropriate, as it has already been used in previous Small Ruminants test evaluation (analytic sensitivity)
- In consequence the EFSA TSE Testing Expert Group in agreement with IRMM proposes that:
 - Evaluation of sensitivity and specificity of tests using positive and negative field scrapie cases should be performed using both tissue slices and macerates.

- Comparison of results obtained with both matrices and follow up of macerate stability will be used for definitive validation of macerate use.
- Because of the reduced number of samples available and/or the need for a direct comparison between assays, BSE in sheep, atypical cases, and preclinical cases and sample dilution investigation will be performed using macerates.

7.3. Procedure for preparation and dispatching of samples

- Tissue slice will be prepared by the IRMM.
- Macerates from the different tissues, will be prepared under the supervision of the IRMM with assistance of CRL and German NRL.
- The protocol for the preparation of such macerates including the dilutions to be used is outlined below (annex 2).
- Liquefied autolyzed field samples will be homogenized by stirring. Macerates will then be aliquoted (500 mg of tissues) before storing at -80°C .
- Dispatching of all samples will remain under the auspices of the IRMM.

8. EVALUATION

The European Commission will organise the evaluation in its practical terms and the IRMM –JRC will carry out and supervise the laboratory evaluations.

8.1. Classical performances parameters

The primary parameters to be evaluated are:

- a. Sensitivity (in clinical and preclinical cases and autolyzed samples);
- b. Specificity;
- c. Assessment of repeatability;
- d. Discriminatory power between positives and negatives;
- e. Robustness of the test (*e.g.* autolyzed samples).

For classical scrapie sensitivity and specificity (discriminatory power) will be calculated using the results obtained from the tissue slice approach.

8.2. Repeatability assessment

Each sample (positives and negatives) will be tested in duplicate into different assays from the same homogenate stage of the test. Both results will be transmitted to the IRMM and only the first result will be taken into account for sensitivity and specificity determination if the test developer specifies that only one replicate has to be carried out.

8.3. Detection limit

Apart from the classical performance parameters, the detection limit of each test will be determined for BSE in sheep, classical scrapie isolates and atypical scrapie.

8.3.1. Analytical sensitivity

- (i) Four BSE in sheep isolates, two ARQ and two ARR.
- (ii) Two – four atypical scrapie samples isolates.
- (iii) Two – four classical scrapie isolates.

All samples will be collected and prepared as macerates by IRMM. The relative detection limit for each test will be analyzed using serial dilutions of this macerates.

8.3.2. Bioassay titration

Macerates for samples sets as defined in 8.3.1 will be homogenized to a final concentration of 10% (tissue weight/vol) in 5% glucose (isotonic solution) and heated for 15 min at 70°C. They will then be serially diluted in negative homogenates (1/10) before inoculation into mice. Mice to be used are:

- Tg Bov (Tg 110 – castilla et al) for BSE in sheep.
- Tg 338 for classical scrapie and atypical scrapie cases.

Titration will be performed using 12 mice per dilution step and isolate. Results obtained will be used to determine the relative sensitivity of each test towards bioassay.

Sample preparation will be performed under the responsibility of the IRMM.

These data will be used to calibrate the test response to a number of infectious particles, which will allow a comprehensive comparison of test performance with reference to biological (gold) standard. However this specific infectivity of a sample would only have relevance to a particular animal bioassay model, and results should not be used to infer the risk of infection following exposure of humans or animals to that sample. Use of the bioassay data will be at the discretion of the experts evaluating test results.

8.4. Re-testing of samples

The companies may be asked by IRMM to re-test samples blindly in combination with others for verification purposes in the event of unexpected low positivity or incorrect categorization of samples. The outcome of the first measurement will be considered as a final judgment and used for analysis unless an obvious sampling mistake has occurred or a series of measurements have been declared invalid by the test developer for obvious technical reasons.

8.5. Data collection, results analysis and Performances Requirement

8.5.1. Data collection, compilation and analysis

All data will be kept confidential until a report with a final conclusion has been drafted by IRMM and a designated EFSA TSE Testing Expert Group. Anonymity of the tests will be kept until conclusions on the performance of the tests have been established by the EFSA TSE Testing Expert Group.

All raw data including the final judgment on a sample must be transmitted daily in an electronic form to the IRMM (provided by IRMM). Commission services staff, being on site in the respective producers' laboratory, will collect one paper copy of the file daily. Laboratory books must be accessible for inspection and original photographs, blots etc. must be made available for analysis to Commission staff. Performance of the test will be analyzed by the EFSA TSE Testing Expert Group with assistance of the IRMM. At this stage anonymity for tests must be warranted.

8.5.2. Performances requirement

In addition to satisfactory robustness and repeatability, a test will only be considered for approval if minimal criteria concerning sensitivity and specificity, as defined below, are met.

8.5.2.1. Clinical BSE and classical scrapie cases

- A 100% detection of positive samples of brainstem slices (macerates will be excluded from this calculation) from confirmed animals is required (fresh and autolyzed). Tests that do not fulfill this requirement should not be recommended for approval.
- For lymph node tissue, sensitivity of the test will only be calculated using samples from lymphoid tissue which was positive by both initial ELISA and WB. Tests that are not 100 % sensitive cannot be approved for use on lymphoid tissue. However, failure on lymphoid tissue does not preclude approval for use on CNS.
- For specificity, a maximum of 4 false positive results out of the 1000 samples is allowed; any tests that have a higher false positive rate should not be recommended.

8.5.2.2. Atypical cases:

Tests that are not able to detect 100% of the positive samples will not be approved.

8.5.2.3. Preclinical cases

Tests that can detect positives in pre-clinical cases should be preferred for approval. This point will be taken into account in the final assessment in a qualitative manner (see Annex 3, Tables 1 and 2).

8.5.2.4. Test sensitivity/Bioassay

New tests and two previously approved tests that were determined to be the most sensitive during the previous evaluation (IDEXX Herdcheck BSE scrapie antigen EIA test and the BIORAD TeSeE sheep/goat test (EFSA, 2005a; EFSA, 2005b)) will be evaluated against the panel of reference materials. A new test will be approved if the limit of detection (*i.e.* detection limit as determined by bioassay) against all the classes of material as specified in chapter 8.3.1 is better than, similar to or no

more than 2 logs poorer than the most sensitive (using bioassay as an external reference) . Outlying results that are inconsistent with an otherwise acceptable performance will be considered by the EFSA WG tests.

8.6. Reporting

Data will be compiled with the shortest possible delay by IRMM and a report on the performance of each individual test will be drafted and submitted for information to the test developers. The data and a final report for each test will be assessed by a scientific working group of the EFSA and transferred after approval to the responsible Commission Service at DG SANCO that is responsible for implementing a decision based on the outcome of the report.

CONCLUSIONS

The BIOHAZ panel concludes:

1. The revised evaluation protocol takes into account experience gained in previous evaluation rounds, knowledge accumulated in recent years with the active surveillance program and constraints such as practicability and potential availability of biological material.
2. New tests have to successfully pass all stages of the evaluation process. Progress to the next stage requires successful completion of the previous stage and therefore the process can be suspended at any stage of the evaluation.
3. This protocol ensures that newly approved tests will not be inferior to previously approved TSE post mortem screening tests.
4. The revised evaluation protocol now considers test performance on detection of classical scrapie, atypical scrapie and BSE in sheep, detection of preclinical cases and limitations posed by analytical sensitivity in comparison with bioassay.
5. Considering data available about abnormal PrP distribution in the three recognized small ruminants TSE forms (BSE, classical scrapie and atypical scrapie) the use of brainstem appears to be the best compromise for detection of all TSE agents in small ruminants. In consequence, officially confirmed (by CRL and NRL) positive/negative brainstem will be used for the evaluation of tests.
6. The criteria in this revised protocol introduce more comprehensive and higher standards than have previously been approved for validation of small ruminant post mortem TSE tests for classical scrapie and BSE as well as for atypical scrapie.

RECOMMENDATIONS

The BIOHAZ panel recommends:

1. Tests already approved for the detection of TSE in small ruminants should be required to confirm their robustness and their ability to fulfil the additional performance requirements (*e.g.* atypical cases and analytical sensitivity).

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2. Tests that are not able to meet requirements for all types of TSE agents (classical scrapie, BSE and atypical scrapie) on known positive samples should not be considered for testing small ruminants in the field.
3. Tests that fail to meet a requirement in a respect of a particular tissue type (lymphoid/CNS) should not be recommended for application on that tissue.
4. Taking into account past experience and currently available data, and in order to facilitate confirmation of atypical scrapie, cerebellum samples should also be collected.
5. Taking into account the experience gained in the TSE test batch testing protocol³ and because knowledge in the TSE field is rapidly evolving, a system of periodic re-assessment of test approval based on both test field performance and evolving EU policy objectives should be considered by the Risk Managers.

³ See Annex 4

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Annex 1. 2001-2005 Field detection of atypical cases using approved Small Ruminants rapid tests:

In a recent work (Maria Nöremark, preliminary published - Nöremark and Hopp, 2006), data on atypical scrapie collected from 26 countries in Europe were analyzed. The reporting of atypical cases is mandatory in the EU since last year; however, the data she analyzed were from 2001 to mid 2005. In this period, 13 European countries reported atypical cases on sheep or goats, and Maria Nöremark worked on 294 cases detected in sheep until June 30th 2005. She discusses different possible biases, among others the definition of atypical cases and differences in the structure of the sampled population and the type of rapid tests used for surveillance as well as confirmatory methods used in the different countries. Among the 294 atypical cases that were studied, 279 (95%) were detected with the test Bio-Rad, 10 (3.4%) with Histopathology/Immunohistochemistry, 2 (0.7%) with Prionics Check Western SR, 2 (0.7%) with modified Prionics Western, and 1 (0.4%) with both Check Western SR and Bio-Rad. These results need to be compared to the distribution of the rapid tests used for the screening of the 1,120,964 sheep: 42% with Bio-Rad, 31% with Prionics or modified Prionics, 18% with Enfer (see also table 1).

Table 1. Tests used in the screening of small ruminants for TSE over the period 2001-2005 and the percentage of positive atypical cases detected by these tests.

Test used *	% of total atypical scrapie cases detected by this method	% of total tests carried out by this method
BioRad TeSeE	95 %	42 %
IHC/Histology	3.4 %	Not used for screening
Prionics Check WB SR	0.7 %	
Modified Prionics check WB	0.4 %	31 %
Enfer	0 %	18 %

* NB data collected 2001-2005 when some of the recently approved tests were not in use

Annex 2. Homogenisation and dilution of brain tissue for preparation of evaluation samples.

INTRODUCTION

Purpose/Scope of this Protocol

- 1.1 To produce samples for use in test evaluations
- 1.2 The material needs to be prepared in a state which allows storage and optimal recovery on laboratory receipt and testing.

SAFETY

- 2.1 Please comply with health and safety requirements.
Materials known or suspected to contain TSEs are to be handled under Category 3 conditions with derogation at all times. Use of a Class 1 cabinet is essential to the safety of the operator and colleagues.
- 2.2 Any existing local SOPs to control laboratory working conditions shall be followed whilst performing this procedure.

Chemicals and reagents

- 20% Chlorox disinfectant
- Chilled sterile distilled water

Animals/Micro-organisms/Cells

- Brainstem or other CNS material from required species. This should be of known TSE status and supported by documented positive or negative diagnosis by IHC, Rapid Test or Western Blot as described in the Opinion.

Equipment

Note: All equipment must be free from previous contact with TSE-contaminated material or autoclaved at 136°C for 18 minutes.

- Class 1 open-fronted safety cabinet. Installed within a Category 3 (CATIII) containment suite with derogation.
- Clothing as appropriate to CATIII suite. As dictated by local COSHH assessment for CATIII suite work.
- Top-pan balance, capable of weighing up to 1000 gm to within 2 decimal places.
- Plastic disposable forceps
- Plastic disposable knife
- Large disposable weighing boats
- Small disposable weighing boats
- Absorbent lining paper for safety cabinet BenchKote or similar
- Autoclave Bag
- Autoclave Tin
- Pipette variable, 1-5ml Gilson or similar
- Pipette Tips As compatible with above pipette
- Vortex Mixer
- Handheld Blender Domestic. Braun, Cooksworks or similar.
- Homogenisation beaker
- Disposable pipette, 30ml Eppendorf or similar
- 100-250ml glass Duran Bottles with plastic lid
- Cryovials Nunc or equivalent
- Suitable printed or Large Bar-coded Labels In the format: BHXXX, OHXXX or CHXXX
- Suitable printed or Small Bar-coded Labels In the format: CBHXXXXX, COHXXXXX, CCHXXXXX, OIEXXXX, BAXXXXXX or OAXXXXXX
- Blue Paper Roll
- Tin foil

PROCEDURE/METHOD

NOTE: Record any comments regarding the sample or the process at the time rather than at the end of the sampling and homogenising process.

Preparation of Database and Database Reference Barcodes

- 4.1.1 Labels and worksheets , entry routines for the laboratory information management system must be available in the laboratory in order to ensure identification and traceability of stock homogenates, dilutions and dispensed vials

Preparation of cabinet & materials

- 4.2.1 Check the safety cabinet is switched on and lined with absorbent paper, place the instrument and autoclave bag within it.
- 4.2.2 Ensure 20% Chlorox and paper roll are close by to mop up any spillages or contamination to the outside of plastic ware, etc.
- 4.2.3 Use the data base to create a record for the page for the sample to be homogenised.
- 4.2.4 Place the brainstem in its unopened container in the cabinet. Place its corresponding Duran bottle next to the cabinet.
- 4.2.5 Confirm the 'Sample reference number' and 'sample ID' corresponds between the sample, Duran bottle and Database.
- 4.2.6 Open the brainstem's container, drain any excess liquid and place the brainstem onto a large disposable weighing boat.
- 4.2.7 If only brainstem is required and it is still attached to the rest of the brain orientate it caudally. Gently free the rostral medulla using forceps and cut through the midbrain to free it.
- 4.2.8 Using the forceps and knife remove any blood clots or connective tissue.

Sample Homogenisation

- 4.3.1 Tare a clean large disposable weighing boat on a Top-Pan Balance. Within the safety cabinet place the material to be homogenised on this weighboat.
- 4.3.2 Carefully re-weigh the weighboat to gain the weight of the material to be homogenised. This may involve moving the material outside the safety cabinet, if so ensure it is placed back inside the cabinet immediately after weighing.
- 4.3.3 Record the sample weight on the Database. If a number of samples are to be combined then record their total weight.
- 4.3.4 Slice the clean material into small cubes, approximately 1cm³, to aid homogenisation.
- 4.3.5 Transfer the material into the container it is to be homogenised within.
- 4.3.6 Add an equal amount of sterile distilled water (1gm = 1ml) to the homogenisation container to create a 1:1 dilution. If any material is stuck to the sides of the container, rinse this off as you transfer the water.
- 4.3.7 Position the homogenising device inside the container so that its blades are completely immersed by the water. If the total volume of tissue and water is less than 40g and splashing is a risk, cover the top of the homogenisation container with sterile tin-foil. Secure it round the rim of the container and the shaft of the homogenising device.
- 4.3.8 Begin homogenising following these steps:
1. 30 seconds at low speed, clean homogeniser blades,
 2. 30 seconds at low speed, clean homogeniser blades,
 3. 30 seconds at low speed.
- Times are approximate and depend on the homogenizer. Use the minimum force and time possible. If necessary, repeat until results are satisfactory. Different homogenisers may require different speed settings
- 4.3.9 Clean the homogenizer as described in section 5 of this protocol
- 4.3.10 Tare the Duran bottle used in step 4.1.2 on a Top-Pan Balance. Place the Duran bottle inside the cabinet and transfer the homogenised material into it. If there are any visible lumps return the homogenate to the homogenising device and blend the sample for a further ½ minute.
- 4.3.11 Weight the Duran bottle and record the final weight of the homogenate on database.
- 4.3.12 Place the homogenate on the vortex mixer for approximately a minute to help remove any surplus air bubbles and ensure the homogenate is thoroughly mixed.
- 4.3.13 If appropriate continue with 4.4 or 4.5 below, if not the sample must be stored in a labeled (bar coded) container at –80°C.

Making a dilution series

- 4.4.1 Plan the volumes of homogenate required to make the dilution series previous to starting work in the CAT III.
- 4.4.2 Follow the homogenisation method above to produce individual sample homogenates, or one positive and one negative homogenate brain pool.
- 4.4.5 First thoroughly vortex the initial homogenised samples and then place them in the safety cabinet. The samples should be of a uniform colour and viscosity.
- 4.4.6 Tare the Duran bottle on a Top-Pan Balance. Place the Duran bottle inside the cabinet and transfer the volumes of homogenate decided upon in step 4.4.1 into it. Weigh the Duran bottle and record the final weight of the homogenate on the database.
- 4.4.7 Thoroughly vortex the new sample to ensure homogeneity.
- 4.4.8 If appropriate continue with 4.5 below, if not the sample must be stored at -80°C in a labelled (bar-coded) container.

Creation of Aliquots

- 4.5.1 From the volume of homogenate weighed in 4.4.4 and the volume of the aliquots to be created, estimate the number of aliquots you will make. Apply appropriately coded labels to each Cryotube.
- 4.5.2 Record creation of aliquots.
- 4.5.3 Using the pipette, aliquot the homogenate into the pre-labelled cryotubes.
- 4.5.4 Aliquots are stored in barcoded tubes at -80°C

Confirmation of Diagnosis

- 4.6.1 Before any of the aliquots can be used for evaluation purposes, randomly chosen representative aliquots from the same preparation must be tested according to the opinion using the following 3 tests: BioRad Sheep and Goat TeSeE assay; Idexx Herd check scrapie antigen test and OIE western blot (FLI protocol) using 0.5g tissue to prepare the samples and monoclonal antibody SHA31 as the anti PrP antibody (or an alternative with equivalent sensitivity)
- 4.6.2 Samples are dispatched with appropriate licences on cardice and packed according to detailed procedures in compliance with IATA regulations.

Update of 'Tissue List'

- 4.7.1 At the end of the homogenising process the database must be updated to reflect the changes made in the level of tissue stocks used.

CLEANING OF THE HOMOGENISER

- 5.1.1 A cleaning procedure has been developed by the CRL which is available on request

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Annex 3. Table 1. PrP^{Sc} immunodetection in the gastro-intestinal tract of susceptible VRQ/VRQ sheep with natural scrapie.

Sheep with a positive reaction in the investigated tissues are indicated as the number of positive sheep out of the three examined at each time point. When less than three sheep were positive, the number of animals is followed by the identification of the positive sheep, arbitrarily named a, b and c. The PrP^{Sc}-immunostaining intensity is indicated as negative (-), rare (+), weak (++), moderate (+++) and heavy (++++). Clinical signs of scrapie were observed in the 23- month-old sheep.

* duo, duodenum ; jeju, jejunum ; ENS, enteric nervous system ; PP, Peyer's patches ; MLN, mesenteric lymph node. ND, not determined.

Organ*	7-month-old		10-month-old		13-month-old		16-month-old		19-month-old		23-month-old	
	Positive sheep	PrP ^{Sc} -staining	Positive sheep	PrP ^{Sc} -staining	Positive sheep	PrP ^{Sc} -staining	Positive sheep	PrP ^{Sc} -staining	Positive sheep	PrP ^{Sc} -staining	Positive sheep	PrP ^{Sc} -staining
Tonsil	3	+++	3	+++	3	+++	3	+++	3	++++	3	++++
Oesophagus:												
- proximal	0	-	0	-	0	-	0	-	0	-	2 (a,c)	++
- mid	0	-	0	-	1 (b)	+	0	-	1 (b)	+	2 (b,c)	++
- distal	0	-	0	-	0	-	0	-	1 (b)	+	1 (a)	+
Reticulum	0	-	0	-	0	-	0	-	3	++	3	++
Rumen	0	-	0	-	0	-	0	-	2 (a,b)	+	2 (a,c)	++
Omasum	0	-	0	-	0	-	1 (b)	+	2 (b,c)	+	2 (b,c)	++
Abomasum	0	-	0	-	2 (b,c)	+	3	++	2 (b,c)	+	2 (b,c)	++
Duodenum	1 (c)	+	2 (a,b)	+	2 (b,c)	+	3	+++	3	+++	3	+++
ENS PP duo	2 (b,c)	++	1 (b)	+	2 (b,c)	++	3	+++	3	+++	3	+++
PP duodenum	3	++	3	+++	ND	ND	3	+++	2 (b,c)	++++	3	++++
Jejunum-25%	0	-	2 (a,b)	+	3	++	2 (a,c)	++	3	+++	3	+++
ENS PP jeju-25	2 (a,c)	++	2 (a,b)	+	3	++	3	+++	3	++	3	+++
PP jeju-25%	3	++	3	++	3	+++	3	+++	3	++++	3	++++
Jejunum-50%	0	-	0	-	3	++	2 (a,c)	++	3	+++	3	+++
ENS PP jeju-50	3	++	3	+	3	++	3	+++	3	+++	3	+++
PP jeju 50%	3	++	3	++	3	+++	3	+++	3	++++	3	+++
Jejunum-75%	0	-	1 (b)	+	3	++	3	++	3	+++	3	+++
ENS PP jeju-75	3	++	3	+	3	++	3	+++	3	+++	3	++
PP jeju 75%	3	+++	3	++	3	+++	3	+++	3	++++	3	+++
Ileon	0	-	2 (a,b)	+	3	++	3	++	3	+++	3	++++
ENS PP ileon	3	++	3	+	3	+++	3	++	3	++++	3	+++
PP ileon	3	+++	3	+++	3	+++	3	+++	3	++++	3	++++
MLN ileon	3	++	3	+++	3	+++	3	+++	3	++++	3	++++
Cæcum	0	-	0	-	2 (b,c)	++	2 (a,b)	++	3	+++	3	+++
ENS PP cæcum	3	++	2 (a,b)	+	3	++	3	++	3	+++	3	+++
PP cæcum	3	+++	3	++	3	+++	3	+++	3	++++	3	+++

Protocol for the evaluation of rapid *post mortem* tests to detect TSE in small ruminants

Table 2. PrP^{Sc} immunodetection in the obex and spinal cord segments of susceptible VRQ/VRQ sheep with natural scrapie

Sheep with a positive reaction in the investigated tissues are indicated as the number of positive sheep out of the three examined at each time point. When less than three sheep were positive, the number of animals is followed by the identification of the positive sheep in brackets, arbitrarily named a, b and c. The PrP^{Sc} -immunostaining intensity is indicated as negative (-), rare (+), weak (++) , moderate (+++) or heavy (++++). Samples from the three 7-month-old sheep were all negative. Clinical signs of scrapie were observed in the 23- month-old sheep. * C, cervical, T, thoracic, L, lumbar and S, sacral spinal cord segments. ND, not determined.

Organ *	10-month-old		13-month-old		16-month-old		19-month-old		23-month-old	
	Positive sheep	PrP ^{Sc} detection	Positive sheep	PrP ^{Sc} detection	Positive sheep	PrP ^{Sc} detection	Positive sheep	PrP ^{Sc} detection	Positive sheep	PrP ^{Sc} detection
Obex	2 (a,b)	+	3	+++	3	+++	3	++++	3	++++
C1	0	-	0	-	0	-	2 (a,b)	++	3	++
C2	0	-	0	-	0	-	3	++	3	++
C3	0	-	0	-	0	-	3	++	3	+++
C4	0	-	0	-	0	-	3	++	3	+++
C5	0	-	0	-	0	-	3	++	3	+++
C6	0	-	0	-	0	-	3	++	3	+++
C7	0	-	0	-	0	-	2 (a,b)	++	3	+++
T1	0	-	0	-	0	-	3	+++	3	++++
T2	0	-	0	-	3	++	3	+++	3	++++
T3	0	-	0	-	3	+	3	+++	3	++++
T4	0	-	3	+	3	++	3	+++	3	++++
T5	1 (b)	+	2 (b,c)	+	3	+	3	++++	3	++++
T6	2 (a,b)	+	2 (a,c)	+	3	++	3	++++	3	++++
T7	1 (a)	+	3	++	3	+++	3	++++	3	++++
T8	1 (b)	+	3	++	3	++	3	++++	3	++++
T9	1 (b)	+	3	+	3	++	3	++++	3	++++
T10	1 (b)	+	3	+	3	++	3	++++	3	++++
T11	1 (b)	+	3	+	3	++	3	++++	3	++++
T12	0	-	3	++	3	++	3	++++	3	++++
T13	1 (b)	+	3	+	3	++	3	++++	3	++++
L1	2 (a,b)	+	1 (b)	+	3	++	3	++++	3	++++
L2	0	-	2 (b,c)	+	3	++	3	+++	3	+++
L3	0	-	0	-	3	+	3	+++	3	+++
L4	0	-	0	-	0	-	3	++	3	+++
L5	0	-	0	-	0	-	3	+++	3	++
S1	0	-	0	-	0	-	1 (a)	+++	ND	ND

Annex 4. Comments on the re-evaluation of currently approved tests for small ruminant TSE surveillance.

Several tests are currently approved for surveillance of TSEs in small ruminants (EFSA, 2005a; EFSA, 2005b). However, in their recent Opinion on the CRL report on batch testing of TSE rapid tests: sample selection and test sensitivity issues⁴, the BIOHAZ Panel came to the following conclusions :

“The experts of the Scientific Panel on Biological Hazards (BIOHAZ Panel) reviewed the CRL report on batch testing data and concluded that not all of the nine tests evaluated performed equally. The implications of this are twofold; firstly, the sample panel cannot be used in its current state to provide a batch testing system for all currently approved EU BSE rapid tests, although it is suitable for most of them. Secondly, they also suggest that there are profound differences in performance in terms of robustness, with respect to sample format, displayed by currently approved rapid tests. Consequentially, any observed differences in performance, if real, would be of concern. The observation that aliquots of the same positive sample were found to be highly positive according to some of the approved rapid tests but negative according to others, could be attributable to aspects of the test performance and/or to properties of the sample material tested.”

These concerns were addressed in a number of recommendations, and recommendation 2, p15 of their report is especially relevant to this revised protocol :

“The BIOHAZ panel recommends further assessment of currently approved tests to detect potential changes in performance with time or between batches, which may affect the usefulness in determining disease/infection prevalence especially in the frame of the present decreasing BSE prevalence”.

In this context, we feel that re-evaluation of currently approved tests, according to the criteria listed herein, is justified.

⁴ For citation purposes: Opinion of the Scientific Panel on Biological Hazards on a request from the European Commission on the CRL report on batch testing of TSE rapid tests: sample selection and test sensitivity issues, *The EFSA Journal* (2007), 443, 1-18.