

Protocol for the evaluation of new rapid BSE *post mortem* tests¹

Scientific Opinion of the Panel on Biological Hazards

(Question No EFSA-Q-2007-053)

Adopted on 7 June 2007

PANEL MEMBERS

Olivier Andreoletti, Herbert Budka, Sava Buncic, Pierre Colin, John D Collins, Aline De Koeijer, John Griffin, Arie Havelaar, James Hope, Günter Klein, Hilde Kruse, Simone Magnino, Antonio Martínez López, James McLauchlin, Christophe Nguyen-The, Karsten Noeckler, Birgit Noerrung, Miguel Prieto Maradona, Terence Roberts, Ivar Vågsholm, Emmanuel Vanopdenbosch.

SUMMARY

Currently, 12 rapid BSE test kits are approved by the EC for the routine *post mortem* testing of slaughtered cattle over 30 months of age in accordance with the TSE Regulation (EC) No 999/2001. The EC is considering launching a new open call for expression of interest for rapid tests for use in the framework of TSE monitoring. Therefore, the EFSA was requested to update the current protocols for the laboratory evaluation and field trial, taking into account experience gained in past evaluation rounds.

The experts of the BIOHAZ panel conclude that the revised evaluation protocol takes into account, experience gained in previous evaluation rounds, knowledge accumulated in recent years with the active surveillance program, constraints such as practicability and potential availability of biological material. This protocol ensures that newly approved tests will not be inferior to previously approved BSE *post mortem* screening tests. The criteria in this revised protocol introduce more comprehensive and higher standards than have previously been approved for validation of cattle *post mortem* BSE tests which will allow a more comprehensive evaluation of the test's performance (analytical sensitivity). Since the last rapid test evaluation two new forms of TSE in cattle have been reported in different EU member states and USA (Atypical BSE H and L). Despite concerns about these two types of TSE, the protocol does not include evaluation of rapid test performances with regards to atypical BSE, because currently available biological material is lacking.

¹ For citation purposes: Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on a protocol for the evaluation of new rapid BSE *post mortem* tests. *The EFSA Journal* (2007) 508, 1-20

Opinion on a protocol for the evaluation of new rapid BSE *post mortem* tests

The BIOHAZ panel recommends that tests previously approved for the detection of BSE in cattle should be required to meet newly performance criteria (*e.g.* analytical sensitivity). When H or L type BSE brain material becomes available, the performance of tests that would have been approved for classical BSE, should be evaluated on this material. In the hypothesis that such tests would not meet performance criteria on BSE H and L types, they should not be considered for field testing. 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: BSE, Bovine Spongiform Encephalopathy, rapid tests, *post mortem* BSE test, evaluation, field trial.

TABLE OF CONTENTS

Panel Members.....	1
Summary.....	1
Table of Contents.....	3
Background.....	4
Mandate.....	4
Acknowledgements.....	5
Design of a protocol for the evaluation of new rapid BSE post mortem tests including laboratory and field evaluation.....	5
1 Introduction.....	5
2 Definitions.....	5
3 Purpose of the EU wide surveillance programme.....	6
4 Purpose of the evaluation.....	6
5 The Evaluation Process.....	6
5.1 Assessment of submitted dossier.....	7
5.2 Pre-evaluation assessment.....	7
5.2.1 Criteria for admission to full evaluation.....	8
5.3 Scope of Evaluation.....	8
5.4 Objectives of the evaluation protocol.....	9
5.5 Laboratory evaluation.....	9
5.6 Field Trial.....	9
5.6.1 Estimation of diagnostic sensitivity relative to approved tests.....	10
5.6.2 Estimation of specificity relative to approved tests.....	10
5.6.3 Variation in sample quality.....	11
5.6.4 Resolution of discrepant results from specificity testing (5.6.2) and from poor quality samples (5.6.3)11	
6 Description of the test procedure.....	12
7 Confidentiality.....	12
8 Criteria for tests to be accepted.....	12
Conclusions.....	13
Recommendations.....	13
Documents provided to EFSA.....	13
References.....	14

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 EU legislation all slaughtered cattle over the age of 30 months have to be tested using one of the approved² “rapid BSE tests”. In addition, a certain sample size of fallen stock over 24 months of age as well as all emergency slaughtered cattle over 24 months of age have to be subjected to an approved rapid test. Presently, twelve test kits from ten manufacturers are available for testing for BSE in cattle, after having been evaluated and subsequently approved by the EC.

MANDATE

The Commission is planning to launch a new open call for expression of interest for rapid test for use in the framework of TSE monitoring. This call is intended to cover tests for TSE detection both in large and small ruminants. In order to achieve this and as preparatory work, there is a need to update current evaluation protocols. These new protocols will be the basis for any future evaluation round of rapid *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.

This document describes the updated evaluation protocol for rapid BSE tests in cattle which should ensure that the performance of any new rapid BSE test is not statistically inferior to that of the currently approved tests. The purpose of such an evaluation is not to rank the sensitivities of approved and new rapid BSE tests or to find out whether the new rapid BSE tests are able to detect BSE in cattle earlier in the incubation period. These issues should be assessed in separate studies.

² As laid down in Annex 10, chapter C to Regulation 999/2001

ACKNOWLEDGEMENTS

The Experts of the working group are acknowledged for their work for this mandate. The Members are: Olivier Andreoletti (Chairman), Jean-Noel Arzac, Thierry Baron, Anne-Gaëlle Biacabe, Martin Groschup, Jim Hope, Peter Lind, Heinz Schimmel (JRC), Emmanuel Vanopdenbosch, Danny Matthews, Angus Wear (rapporteur), Katherine Webster.

Design of a protocol for the evaluation of new rapid BSE *post mortem* tests including laboratory and field evaluation

1 INTRODUCTION

The Commission is planning to launch a new open call for expression of interest for rapid test for use in the framework of TSE monitoring. This call is intended to cover tests for TSE detection in both large and small ruminants. In order to implement this project and as a preparatory work, there is a need to update current evaluation protocols. The 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 the previous evaluation rounds.

For this purpose the “Opinion on a design of a Field Trial for the Evaluation of New Rapid BSE *post mortem* Tests” adopted on 22 February 2002 (SSC, 2002) and “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” (EFSA, 2004a) serve as a basis for the establishment of a modified protocol for such an evaluation. This protocol requires reference to the preceding documents for some of the technical and background detail.

2 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 brainstem material 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

Opinion on a protocol for the evaluation of new rapid BSE *post mortem* tests

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 1 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.

3 PURPOSE OF THE EU WIDE SURVEILLANCE PROGRAMME

In 1999 and 2003 the EU (Scientific Steering Committee (SSC) and later the EFSA both in cooperation with the Joint Research Centre (JRC) and its Institute for Reference materials and measurements (IRMM)) evaluated rapid tests for TSE epidemio-surveillance in cattle. The evaluation process was conducted following recommendations from scientific experts and reflected the knowledge in 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.

4 PURPOSE OF THE EVALUATION

The purpose of the review of the protocol for TSE test evaluation in cattle is to define the evaluation criteria to be used, taking account of the detection of new strains during on-going surveillance, based on acquired knowledge from past evaluations, and assuring continuity and the use of an approach that remains practical. Manufacturers who have a test ready for use under field conditions, together with properly documented validation and quality data to support the application are invited to apply. This evaluation is not appropriate for tests still undergoing development or those intended solely as research tools.

5 THE EVALUATION PROCESS

Evaluation of TSE tests submitted following a call for expression of interest launched by the EC comprises different steps. These include the assessment of the submitted dossier and a pre-evaluation assessment by the EFSA TSE Testing Expert Group. Further steps cover a laboratory evaluation and a field trial which are both supervised by personnel of the IRMM, also responsible for the sample distribution, data collection and analysis, and reporting. Evaluation of the IRMM report as well as the preparation of the package insert is carried out by EFSA TSE Testing Expert Group. A test may be excluded from further assessment at any

stage of the process. Reference is made to the previous protocol (EFSA, 2004a)³ for more background and details on the field trial.

5.1 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 CNS samples. 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 the samples if macerates are used).
- b. Details of the method used to test samples and confirm status, where and when this testing was done.
- c. Details of the manufacturers' quality system with a copy of the scope of accreditation, if currently available.
- d. Any additional information on demand of the EC on test set up and performance.

5.2 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 5.1 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 also include a panel of dilution series. All samples will be prepared as macerates. The relative detection limit for each test will be analyzed using serial dilutions of macerate.
- d. This will allow the inspectors to make a preliminary assessment of the method, and compare performance with manufacturers' claims.
- e. The dilution series will be made from macerates of classical BSE 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 (dilution series prepared by the manufacturer from macerates already validated with the highest sensitive test; see previous EFSA reports). A new test will be approved if the limit of detection is better than, similar to or no more than 2 logs poorer than the most sensitive. Outlying results that are inconsistent with an otherwise acceptable performance will be considered by the EFSA TSE Testing Expert Group.

³ EFSA Scientific Report 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 protocol for the evaluation of new rapid BSE *post mortem* tests

Equivalent samples will have been subjected to prior testing with an ELISA test having shown high analytical sensitivity performance on previous evaluation (EFSA, 2004b)⁴. For all positive samples, a confirmatory WB aiming at profile identification will have been carried out (using 0.5 grams tissue using anti-PrP antibody with at least equivalent sensitivity as with 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.

5.2.1 Criteria for admission to full evaluation

The criteria for passing into the full evaluation will be:

- A satisfactory assessment of the submission dossier,
- 100% performance on the proficiency test panel.
- A new test will be approved for full evaluation if the limit of detection is better than, similar to or no more than 2 logs poorer than the most sensitive test. Outlying results that are inconsistent with an otherwise acceptable performance will be considered by the EFSA TSE Testing Expert Group.
- An acceptable visit.

5.3 Scope of Evaluation

During the full evaluation, the submitted tests will be evaluated using bovine brain tissue collected during active and passive surveillance programmes. EFSA acknowledge that new forms of BSE have now been detected (type L and H) (Béringue *et al.*, 2006; Biacabe *et al.*, 2003; Biacabe *et al.*, 2007; Casalone *et al.*, 2004; Jacobs *et al.*, 2007; Richt *et al.*, 2007; Yamakawa *et al.*, 2003). It is valuable to notice that already identified atypical BSE cases were detected by a range of already approved field tests. Due to the scarcity of this material, samples of these variants are not expected to be included within the sample set for this evaluation. However, when such material becomes available, additional testing of such samples is recommended. Therefore, EFSA may request additional testing of such samples at any stage.

With regard to work already performed on classical BSE, and in contrast to experience with scrapie, there appears to be no apparent difference between testing methods in terms of their capacity to detect BSE. In consequence, there is less need to carry out a detailed investigation of sensitivity involving bioassay for BSE (in contrast to the new protocol for small ruminants).

It is envisaged that successful tests will be approved for testing only in accordance with the test parameters and test components specified in the evaluation *i.e.* the protocol used during the evaluation.

⁴ EFSA Scientific report on the evaluation of seven new rapid post mortem BSE tests, adopted on 16th November 2004.

5.4 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. Detection limit of each diagnostic test (analytical sensitivity).

This last point is considered to be a critical point for the key issue of public health and for the capacity of a test to detect BSE in cattle 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

5.5 Laboratory evaluation

Each manufacturer will test in their own or a chosen laboratory a panel of samples for evaluation. This testing will be supervised by an EFSA/ IRMM approved person.

The samples will include the following:

- 50 positives – 200 negatives (tissue slices)
- An analytical sensitivity series (prepared from macerates and further processed according to the manufacturer protocol)

Reference is made to the previous IRMM report on the evaluation of rapid post mortem tests (IRMM, 2004)⁵.

5.6 Field Trial

In addition to a manufacturers laboratory based evaluation of anonymous positive and negative samples the final evaluation will include a field trial of each test. The field trial stage of the evaluation will provide manufacturers the opportunity to apply their tests to both fresh and autolyzed samples as tested in routine BSE testing laboratories within the EC. Further sensitivity testing will be performed with confirmed positive material to be tested within national reference laboratories (EFSA report of details)⁶.

⁵ Report on The evaluation of 10 rapid post mortem tests for the diagnosis of transmissible spongiform encephalopathy in bovines. W Philipp, P v Iwaarden, M Goll*, N Kollmorgen, H Schimmel, P Vodrazka. 08 October 2004.
(http://www.irmm.jrc.be/html/activities/TSE_testing/phaseIBSEtestevaluation2004globalreport.pdf)

⁶ EFSA Scientific Report on the Design of a Field Trial Protocol for the Evaluation of New Rapid BSE post mortem Tests (Adopted on 5 April 2004).

5.6.1 Estimation of diagnostic sensitivity relative to approved tests

Sensitivity is the probability that a test recognises confirmed positive test specimens (“true positives”) as positive. Ideally, there should be no “false negative” test results, *i.e.* the sensitivity is 100 %.

In order to demonstrate that the sensitivity of the new rapid test is not inferior of the sensitivity of an approved test (99 %- IC 95 %), the sample size has to comprise at least 138 (258) samples that are positive by one of the approved tests. Samples, which are not detected positive by the approved test, are excluded from the study. All of the remaining samples have to be recognised by the new rapid test as positive. Any sample which tests negative with the new rapid test must be re-tested in duplicate from the original sample preparation. Both re-test results must be positive (2 out of 3 have to be positive). The original preparation of the sample must also be re-tested in duplicate with the approved test. Two negative results with the approved test will exclude the sample from the study.

Each test manufacturer will be provided with **200 true positive samples** to be tested by the new rapid test, which would ensure with a 95 % probability that the sensitivity of the new rapid test is not below 98.5 % compared with the approved test (see above).

All samples provided will have a known and well documented provenance.

In order to avoid test results being discrepant between any new rapid test and approved tests due to an uneven distribution of prion proteins the samples will be made homogeneous (macerates). A protocol for the maceration treatment which has no or minimal influence on subsequent steps has been developed by the EU Community Reference Laboratory. A sufficient number of aliquots providing enough material for one test (500 mg brain stem) will be prepared for this purpose. At least one aliquot will be archived. All sample homogenates will be previously checked against two approved tests.

The tests will be performed in NRLs of a Member State (or Switzerland). Derogating from the general rule that sensitivity testing should be carried out in one or more NRL(s), certain other laboratories may take part in the study if the respective NRL does not conduct the study itself and if the responsible NRL agrees.

At least two batches of the test kits should be included. It is the responsibility of the company, which intends to market the new rapid test, to select a NRL (or following the derogation a state owned laboratory). The company should compensate the laboratory performing the comparisons for all expenses.

Testing of the samples with the new assays should be executed by local staff, or, at the discretion of the NRL, by the test developer’s staff under supervision of local NRL staff.

IRMM will be notified on the initiation of such studies (where the tests will be carried out; number of samples; which approved tests have been chosen for comparison) and will collect and evaluate the data. The raw data will be communicated to IRMM on completion. The IRMM will provide a standardised data format.

5.6.2 Estimation of specificity relative to approved tests

Specificity is the probability that a test recognises truly negative test specimens as negative. Ideally, there should be no “false positive” test results, *i.e.* the specificity is 100 %.

Opinion on a protocol for the evaluation of new rapid BSE *post mortem* tests

For practicability, it was decided that **10,000 consecutive samples** from healthy slaughtered animals (see 5.6.3 for exceptions to this) that are tested negative using an approved test should be used for the estimation of specificity in comparison with approved tests.

The tests should be performed with the agreement of the NRL in experienced high throughput routine laboratories. The samples should be prepared according to the company's protocol. Fresh material immediately adjacent to the usual sampling region can be used for the comparison of the tests. Laboratories of at least two Member States (or one Member State and Switzerland) will be involved at the discretion of the company. The maximum proportion of samples tested in a single laboratory should not exceed 70 % of all samples. At least two batches of the test kits should be included.

Each new rapid test should be compared to at least two approved tests. This comparison has not necessarily to be done in parallel. No more than 70 % of the samples should be compared against one approved test.

It is the responsibility of the company, which intends to market the new rapid test to select the laboratories as well as the approved tests used for comparison. The two approved tests chosen for comparison must not be from the same manufacturer. The company should compensate the Laboratory performing the comparisons for all expenses.

The NRL will be notified on the initiation of such studies. It will take adequate measures to avoid a disturbance of national statistics.

Discrepant results between the new rapid test and the approved test(s) will be resolved by the responsible NRL and the EU Community Reference Laboratory (CRL) in Weybridge according to an algorithm described below. Confirmed positive results will be excluded from the calculation of specificity.

IRMM will be notified of the detail and initiation of such studies. The raw data will be communicated to IRMM on a daily basis for analysis. The IRMM will provide a standardised data format.

5.6.3 Variation in sample quality

In practice, many samples to be tested will be of poor quality (*e.g.* autolysed or putrified). Therefore, it has to be demonstrated that such conditions do not bias new test performance relative to approved test performance (Wear *et al.*, 2005). The positive samples will inevitably contain a proportion of poor quality material. A minimum of 1,000 poor quality samples must be included within the total of 10,000 negative samples tested for specificity. These must be compared with an approved test at VLA Newcastle or an NRL. All samples will be graded to indicate the level of autolysis with a minimum of 70% categorised as being “grossly autolysed with some retention of structure” (or worse). Clarification of sample grading can be provided by the Community Reference Laboratory. IRMM will be notified on the initiation of such studies and will collect and evaluate the data. The raw data will be communicated to IRMM on a daily basis. The IRMM will provide a standardised data format.

5.6.4 Resolution of discrepant results from specificity testing (5.6.2) and from poor quality samples (5.6.3)

Two approaches will be used:

- a. A homogenised sample preferentially prepared according to an IRMM approved protocol will be prepared by the responsible NRL from the brain stem which gave rise to the discrepant results. Samples of the homogeneous material should be re-tested with the two tests in question by the laboratory where the discrepant results

Opinion on a protocol for the evaluation of new rapid BSE *post mortem* tests

were produced. This approach should be used to resolve discrepant results possibly due to the uneven distribution of the abnormal prion protein in the initial samples.

- b. Confirmation will be performed at the CRL according to their established procedures.

6 DESCRIPTION OF THE TEST PROCEDURE

A number of laboratories will be involved in the evaluation of the new rapid test. It is essential that their results are comparable. It is therefore necessary to have clear, stringent and detailed descriptions of the test procedures. It is the duty of the companies to provide such descriptions (detailed kit protocols) and to ensure that they are understood in exactly the same way in all participating laboratories.

After the field trial is finished the laboratories involved should meet in order to discuss whether the written test procedures used during the field trials proved to be accurate and fully understandable. Feedback from this discussion should be forwarded to IRMM for inclusion in their report. If necessary, test procedures may have to be clarified.

The clarified test procedures or if clarification is not necessary, the test procedures used, will then be defined as part of the approval. Later changes in the test procedure without consent by the EU Commission services will invalidate the approval.

7 CONFIDENTIALITY

All data generated during the field trial must be kept confidential and should not be made accessible to third parties other than the test developer, the concerned National Reference Laboratory, the testing laboratory, the European Food Safety Authority and the European Commission.

8 CRITERIA FOR TESTS TO BE ACCEPTED

The data will be compiled and analysed by the IRMM. A final report will be prepared and this report will be evaluated by the EFSA Scientific Expert Working Group on TSE Testing. The statistical analysis will be designed to demonstrate non-inferiority of the new rapid test to the already approved tests.

Tests must not perform worse than previously approved tests. A new test will be approved if its performance against the reference and field material is as follows

- a) Successful completion of the initial dossier and pre-evaluation phase,
- b) During the laboratory phase of the evaluation: no false negatives in 50 confirmed positive samples and no more than one false positive in 200 negative samples
- c) In the pre-evaluation and the laboratory evaluation: analytical sensitivity must not be lower than a difference of two logs from the highest sensitivity assay of existing approved tests.
- d) In the field part of the evaluation: no more than 1 false negative in 200 confirmed positive samples (5.6.1.) and no more than 5 false positive tests in 10,000 samples (5.6.2 & 5.6.3).

Each test manufacturer must also seek approval of test instructions (kit insert or IFU) and obtain approval of the quality system by the EU CRL prior to listing in the EC regulation.

CONCLUSIONS

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 BSE post mortem screening tests.
4. The criteria in this revised protocol introduce more comprehensive and higher standards than have previously been approved for validation of cattle post mortem BSE tests which will allow a more comprehensive evaluation of the test's performance (analytical sensitivity).
5. Since the last rapid test evaluation two new forms of TSE in cattle have been reported in different EU member states and USA (Atypical BSE H and L). Despite concerns about these two types of TSE, the protocol does not include evaluation of rapid test performances with regards to atypical BSE, because currently available biological material is lacking.

RECOMMENDATIONS

The BIOHAZ panel recommends:

1. When H or L type BSE brain material becomes available, the performance of tests that would have been approved for classical BSE should be evaluated on this material. In the hypothesis that such tests would not meet performance criteria on BSE H and L types, they should not be considered for field testing.
2. Tests previously approved for the detection of BSE in cattle should be required to meet additional performance criteria (*e.g.* analytical sensitivity).
3. Taking into account the experience gained in the TSE test batch testing protocol⁷ 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.

DOCUMENTS PROVIDED TO EFSA

Letter with the ref. D(2003)CB/MG/ac/310087 from the European Commission – Health & Consumer Protection Directorate-General, requesting to take on the responsibility for the scientific aspects of the evaluation of rapid TSE/BSE tests.

⁷ See attached Annex 2.

REFERENCES

- Béringue, V., Bencsik, A., Le Dur, A., Reine, F., Lai, T.L., Chenais, N., Tilly, G., Biacabé, A-G., Baron, T., Vilotte, J-L. and Laude, H. (2006)** Isolation from cattle of a Prion Strain Distinct from that causing Bovine Spongiform Encephalopathy. PLoS Pathogens. 2. Issue 10. e112. 0956-0963.
- Biacabe, A-G., Laplanche, J-L., Ryder, S.K and Baron, T. (2003)** Distinct molecular phenotypes in bovine prion diseases. EMBO Repts. 5. 110-114.
- Biacabe, A-G., Jacobs, J.G., Bencsik, A., Langeveld, J.P.M and Baron, T.G.M. (2007)** H-type Bovine Spongiform Encephalopathy. Complex molecular features and similarities with Human Prion Diseases. Prion 1:1. 61-68.
- Casalone, C., Zanusso, G., Acutis, P., Ferrari, S., Capucci, L., Tagliavini, F., Monaco, S and Caramelli, M.(2004)** Identification of a second bovine amyloidotic spongiform encephalopathy: Molecular similarities with sporadic Creutzfeldt-Jakob Disease. Proc NY Acad Sci. USA. 101.3065-3070.
- EC (2001)** Regulation 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. O. J. L 147 of 31.05.2001, pp. 1-40.
- EFSA (2004a)** Scientific Report on the Design of a Field Trial Protocol for the Evaluation of New Rapid BSE *post mortem* Tests. EFSA Scientific Report, (1), 1-10.
- EFSA (2004b)** Scientific report on the evaluation of seven new rapid *post mortem* BSE tests. EFSA Scientific Report, (18), 1-13.
- IRMM (2004)** Report on The evaluation of 10 rapid post mortem tests for the diagnosis of transmissible spongiform encephalopathy in bovines. W Philipp, P v Iwaarden, M Goll*, N Kollmorgen, H Schimmel, P Vodrazka. 08 October 2004
www.irmm.jrc.be/html/activities/TSE_testing/phaseIBSEtestevaluation2004globalreport.pdf
- Jacobs, J.G., Langeveld, J.P.M, Biacabe, A-G., Acutis, P-L., Polak, M.P., Gavier-Widen, D., Buschmann, A., Caramelli, M., Casalone, C., Mazza, M., Groschup, M., Erkens, J.H.F., Davidse, A., van Zijderveld, F.G and Baron, T. (2007)** Molecular discrimination of atypical bovine spongiform encephalopathies from a wide geographical region in Europe. J. Clin. Microbiol. On line at jcm.asm.org
- Richt, J.A., Kunkle, R.A., Alt, D., Nicholson, E.M., Hamir, A.N., Czub, S., Kluge, J., Davis, A.J, and Hall, SM. (2007)** Identification and characterization of two bovine spongiform encephalopathy cases diagnosed in the United States. J. Vet. Diagn. Invest. 19. 142-154.
- SSC (2002)** Opinion on the design of a field trial for the evaluation of rapid BSE *post mortem* tests. Adopted by the Scientific Steering Committee at its meeting of the 22nd of February 2002. http://ec.europa.eu/food/fs/sc/ssc/out246_en.pdf.
- Walter, S. D., Irwig, L. M. (1988)** Estimation of test error rates, disease prevalence and relative risk from misclassified data: a review. J. Clin. Epidemiol. 41(9): 923-937.
- Wear, A., Henderson, K., Webster, K., and Patel, I., (2005)** A comparison of rapid bovine spongiform encephalopathy testing methods on autolyzed bovine brain tissue. J. Vet. Diagn. Invest. 17 99-102.
- Yamakawa, Y., Hagiwara, K., Nohtomi, K., Nakamura, Y., Nishijima, M., Higuchi, Y., Sato, Y., Sata, T. and the Expert Committee for BSE Diagnosis, Ministry of Health, Labour and Welfare of Japan (2003)** Atypical proteinase K-resistant prion protein (PrP^{res}) observed in an apparently healthy 23-month-old Holstein steer. Japanese. Journal of Infectious Diseases., 56, 221-222.

Annex 1. **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, Cookworks 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 instruments 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 it's 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 Aliquot 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

Annex 2. Comments on the re-evaluation of currently approved tests for cattle TSE surveillance

Several tests are currently approved for surveillance of BSE in cattle. However, in their recent Opinion on the CRL report on batch testing of TSE rapid tests: sample selection and test sensitivity issues⁸, 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.

⁸ 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.