EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions

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

OPINION

BACKGROUND

According to regulation 270/2002 more than 560.000 small ruminants in the European Union will be subjected to rapid testing for the presence of transmissible spongiform encephalopathy (TSE) per year. Three rapid tests (Enfer, BioRad and Prionics) are approved for the *post mortem* diagnosis of BSE in cattle. In addition to these 3 tests, 5 new tests for rapid BSE diagnosis are under evaluation and the first phase (laboratory evaluation) was finalised in 2001[1] using bovine material.

The Institute for Reference Materials and Measurements (IRMM) of the Directorate General Joint Research Centre acting on behalf of Directorate General for Health and Consumer Protection (DG SANCO) will conduct an evaluation of up to 8 rapid test systems that all have undergone a laboratory evaluation study for their use on bovine brain tissues.

MANDATE

As a follow-up to the suggested Scientific Steering Committee (SSC) strategy of 4 - 5 April 2002[2] to 'investigate the possible presence of BSE in small ruminants' Commission Services invited the SSC to prepare a protocol for the evaluation of rapid tests for the diagnosis of TSEs in small ruminants.

CONCLUSION

The SSC recommends to use the outlined protocol hereafter for the evaluation of rapid *post mortem* tests on tissues of small ruminants. The SSC strengthens the fact that this programme serves only as a first step towards the investigation of the presence of BSE in small ruminants under field conditions. This opinion is based on a programme developed by the TSE/BSE *ad hoc* group.

[1] Report on the evaluation of five rapid tests for the diagnosis of Transmissible Spongiform Encephalopathy in bovines (2nd Study), 27 March 2002

[2] Suggested strategy to investigate the presence of BSE in small ruminants (adopted on 04-05 April 2002)

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REPORT

ON A PROGRAMME FOR THE EVALUATION OF

RAPID *post mortem* TESTS

TO DETECT TSE IN SMALL RUMINANTS

PREPARED BY THE TSE/BSE *AD HOC* GROUP

AND FINALISED AT ITS MEETING OF 10 OCTOBER 2002

1. GENERAL CONDITIONS

The tests will be evaluated for one or more tissues (brainstem, spleen, mesenteric lymph nodes) depending on the requirements of the participants.
It is envisaged to approve successful tests for:

i) the tissues used in the evaluation
ii) test parameters and test components specified in the evaluation.

The participants must provide:

- the threshold values, whether fixed or calculated per experiment
- the definition and calculation of threshold values
- the composition of main solutions (e.g. buffers)
- description of hardware, instruments and disposables used for the test
- the characterisation of antibodies, aliquots of antibodies for possible verifications for test composition
- a precise and finalised test manual
- any additional information on demand of the EC on test set up and performance
- definition of a possible stable control material for batch release control
- specification of the negative and positive controls used in the assay

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

2. SAMPLING

The neuroanatomical distribution patterns of the lesions of the TSE are subject to variation according to a number of host and agent factors. In BSE the uniform pattern of neuropathological changes and the phenotype of disease on primary transmission to mice argues that these major host and agent factors are constant. The conclusion that the BSE epidemic has been due to a single, stable, cattle adapted strain of a scrapie-like agent has had marked implications for the reproducibility of diagnostic approaches, enabling optimisation of sampling of brain tissue for confirmatory diagnosis in clinical cases to be directed at the medulla oblongata (Wells et. al., 2000). By contrast, in natural sheep scrapie it is accepted that variations in the disease phenotype occur according to differences in breed, agent strain and host PrP genotype. Nevertheless, cumulative experience has indicated that in sheep scrapie, the histopathological diagnosis can be made in the majority of clinical cases also by examination of the medulla at the obex (Wood et al. 1997, Wells, 2000). However, information on the variation in distribution of PrP^{Sc} in sheep with scrapie, on which rapid test diagnosis relies, is limited. Furthermore, subdividing brain tissue for application of PrP detection requiring fresh tissue, as required for rapid tests and confirmatory Western Blotting, and fixed tissue, as required for histopathological and PrP immunohistochemical examinations, is subject to the compromise that precisely the same area cannot be used for both techniques.

In general, the colocalisation of vacuolar changes and PrP^{Sc} by immunohistochemistry, particularly in the dorsal nucleus of the vagus nerve (van Keulen et al 1995, Foster et al. 1996) and further indications that this site may be the earliest to accumulate PrP^{Sc} (at least in some forms of scrapie) (van Keulen et al. 2000, Andreoletti et al. 2000 Ryder et al. 2001), provide a justification for the selection of the medulla region for sampling for PrP^{Sc} detection.

A knowledge of pathogenesis relative to phenotype of disease is clearly required in sheep scrapie if appropriate tissues are to be sampled for detection of preclinical disease.

Thus, in addition to the major obstacles for the production of equivalent sample sets experienced with the evaluation of rapid tests for BSE diagnosis, there are further difficulties in relation to such evaluations for scrapie of sheep. Phenotypic variation in the disease and the smaller size of the ovine brainstem, in particular, will contribute to the difficulty of achieving sample sets with a uniform analyte concentration. The structure, the size, the limited amount of a tissue sample as well as the uneven distribution of prions in the respective material are major obstacles for the production of equivalent sample sets. These are the major reasons that aggravate the evaluation of rapid tests for TSE. The fact that no standardised material for calibration of samples and tests exist yet requires the use of field samples from healthy and confirmed TSE affected animals in an evaluation.

Acknowledging these difficulties a random sampling scheme must enable the subdivision of the brainstem into quasi equivalent sets of sub-samples. Given current information it is proposed to apply an established rigorous scheme used earlier in a similar way on material from cattle (Schimmel et al. 2002).

Homogenisation, being the most obvious solution for overcoming the above mentioned heterogeneity, has to be avoided because of its adverse and partially discriminatory effects on the performance of rapid tests including those which have been already approved for BSE testing. Effects observed have been elevated baseline signals leading to false positives and general or specific reduction of the test signals depending on the rigour of the homogenisation procedures used.

2.1 Origin of samples

Positive samples (brainstem, spleen, mesenteric lymph nodes) will be collected from naturally infected sheep which showed clinical signs of scrapie. They originate from several EU member states and Cyprus. In each case the disease was confirmed by an OIE approved diagnostic method, usually histopathology or immunohistochemistry. The PrP geontype of all animals will be determined. Samples are frozen after collection and stored until and immediately after sub-sampling at –70° C. For brainstem samples which have been stored for 2 years at –20 ºC false positive results were obtained with one rapid test for BSE evaluated in 2001 and should therefore be avoided.

Negative samples were collected from healthy sheep in New Zealand and stored in isolated freezers at –70 C. These animals had an age in the range of 4 to 11 years, they mainly (93%) belong to the Romney breed. These sheep were not genotyped but blood samples of each animal are available and will be used for genotyping. Samples for the evaluation will be provided from brainstem, spleen and mesenteric lymph nodes.

2.2. Preparation of samples

Frozen non-homogenised tissues from brainstem, spleen and mesenteric lymph nodes will be offered to perform the assessment. The sample size will be approximately 500 mg allowing a possible re-testing of a sample.
Sub-sampling of brainstem pieces will be done according to an established rigorous permutation scheme applied earlier in a similar way on material from cattle (provided by IRMM). This scheme will ensure equal portions of sample sets from the various locations in a set as previously and in addition equal portions of samples from the contralateral side of the brain being tested with the other tests evaluated. Samples will be prepared and dispatched to the participants by the Joint Research Centre, IRMM, Geel, Belgium.

3. EVALUATION

No commutability of the sheep samples is yet proven though one must consider a precise permutation scheme to distribute the sub-samples in an optimal way. This allows the best possible approximation to quasi-equivalent sample sets. A full equivalence cannot be achieved due to the individual variation in concentration and distribution of PrP^{Sc} in the

tissue and due to the limited size of each tissue batch. Hence, statistics applied on results obtained with homogenous material have only limited power on tissues with a heterogenous distribution of the analyte.

Based on experience gained from earlier evaluations one must concentrate on the estimation of sensitivity and specificity, or alternatively, the determination of a relative detection limit of a diagnostic test. The detection limit is a critical point for the key issue of public health in the case of BSE and will be important for the capacity of a test to detect PrP^{Sc} in early stages of disease in small ruminants. It defines the relative performance of a test and shall be deemed an extremely important parameter for the evaluation of diagnostic tests, however, discrimination of tests due to inappropriate sample properties has to be excluded, i.e. samples need to be commutable.

Precise parameters need to be applied on sampling and storage to decrease deteriorating physical and chemical effects on the samples.

3.1. Parameters

The primary parameters to be evaluated are:

i) sensitivity
ii) specificity
iii) detection limit
iv) discrimination of negative and positive populations.

Additional factors that could be assessed to specify the capacity of a test:

i) possible automation
ii) time to receive results
iii) quantitative results
iv) repeatability
v) application of a positive and negative control sample per run/plate for additional internal quality control

3.1.1. Sensitivity

A test should detect ideally 100% of all positive specimens as positive. Since none of the tests has already been evaluated on small ruminant tissue it is not possible to fix an absolute minimal requirement based on comparison to standards. It became clear during earlier evaluations that many factors like sub-sampling, mechanical, chemical and physical influences, the uneven distribution of PrP^{Sc}, the conservation status of the sample etc. can have an influence on the actual signal. In addition, it was observed that bovine brainstem samples from histologically confirmed BSE cases raised very weak signals in more than one of the test systems. The homogenisation 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. These influences need consideration when trying to determine the true performance of a test.

The use of 300 well characterised positive samples (so far 250 guaranteed) to demonstrate the sensitivity of a test for TSE testing is sufficient and meets the requirement by OIE (Office International des Epizooties, Paris, France) for the evaluation of qualitative diagnostic tests. Additional 50 autolysed samples from confirmed positive cases should be evaluated in a field trial. Autolysed field samples are preferred versus samples autolysed under controlled conditions in a laboratory because of the obvious differences in the properties of the resulting samples. Liquified autolysed field samples will be homogenised by stirring and aliquoted. Keeping in mind the provision of quasi-equivalent sample sets this allows to achieve a confidence value greater than 99,8% as it was applied in the first BSE *post mortem* test evaluation 1999.

3.1.2. Specificity

Defines the probability of a test to recognise truly negative samples as negative. An exact specificity of 100% cannot be proven, however, the specificity should be tested with a sufficiently high number of negative samples. In conformity with the situation in 1999, 1.000 negative New Zealand sheep samples should be tested.

3.1.3. Re-testing of samples

The companies may be asked by IRMM to re-test samples blindly in combination with others for verification purposes in case weak or false identification was assigned. The outcome of the first measurement will be considered as a final judgement 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.

3.1.4. Detection limit

The relative detection limit for 5 BSE rapid tests was analysed using serial dilutions of a titrated positive homogenate mixed on site with a pool of negative brainstem material. This approach appeared to be advantageous compared to refrozen homogenates prepared in advance at IRMM. The freshly prepared aliquots resulted on average in a 3 times higher signal than the refrozen samples.

However, the determination of the detection limit of a test allows the reduction of the sample size of the positive population

If the distribution of the negative and positive samples population is known (possibly to be expressed in dilutions of a common arbitrarily chosen material) and discriminatory effects as observed during the evaluation of BSE rapid tests carried out in 2001 can be excluded it is in principle sufficient to determine the same or lower limit of detection of a test compared to another one to ensure equal or respectively better performance.

However a lower number of positive samples would still be needed to verify the validity of the distribution respectively the concept. In order to describe the detection limit being an important parameter of the performance characteristics at least 0.5 g of a scrapie infected sheep brain stem macerate will be provided by IRMM. In addition negative brain stems will be made available for the production of serial dilutions ensuring the same matrix background composition. The serial dilutions (3 fold dilution steps down to a 1 in 243 dilution and 2 fold for the lower dilution steps) will be made in the laboratory of the test developer according to its optimised procedure. Samples will be coded on site by IRMM staff to ensure objectivity. Each dilution should be split in 4 aliquots and each aliquot should be analysed at least in five replicates.

In addition samples from the opposite side of scrapie positive brain stems, one side being intact tissue the other side being macerated, will be delivered allowing the assessment of possible negative impacts of maceration on the test performance.

4. ANALYSIS

All data will be kept confidential until a report with a final conclusion has been drafted by IRMM in agreement with a designated scientific working group of the SSC.

4.1.Data analysis

All raw data including the final judgement 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.

4.2. Report

Data will be analysed within the shortest possible delay by IRMM and a report on the performance of each single test is drafted and submitted for comments to the test developers. The data and a final report of each single test is assessed by a scientific working group of the SSC and transferred for approval to the responsible Commission Service at DG SANCO that implements a decision based on the outcome of the report.
A summarised report can be drafted and published by IRMM also taking into consideration new scientific aspects for the design and organisation of forthcoming TSE test evaluations.

4.3 Minimal request

A test will be considered for approval if the minimal standards are fulfilled. These minimal standards will be defined by a scientific working group of the SSC and should focus on:

- a defined sensitivity within a defined confidence range
- a defined specificity
- a given discrimination and performance on autolysed samples.

5. STATISTICAL BACKGROUND

Referring to annex 1 of the SSC opinion of 22 February 2002[3] sensitivity and specificity of a diagnostic test should ideally be determined in comparison to a given 'gold standard'. The arguments pointed out above explain why no such 'gold standard' is available yet for rapid TSE tests and therefore applied performance estimation is needed. Again, today no test has ever been evaluated for its application on ovine tissues implying that a pairwise comparison to an established test is not possible.

OIE recommends a sample size for the evaluation of diagnostic tests of 300 positives and 1000 negatives. This allows to reach a sensitivity of >99,2% assuming the test screens a population with at least 1000 positive animals.

A lack in specificity leads to the detection of higher numbers of false positives causing undesired secondary effects. Nevertheless, these samples will be subjected to further analysis where confirmation would consequently fail. A 100% success in testing 1000 samples leads to a specificity of 99,8% within a 95% confidence range.

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