Animal &
Plant Health
Agency

TSE type and strain characterisation in small ruminants

A technical handbook

Version 3.0

June 2026

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1. Introduction

Purpose

To document the properties which can be used to distinguish small ruminant TSE isolates requiring further investigation.

To provide sampling and testing strategies and protocols for discriminatory methods.

To identify those isolates which have the characteristics of the BSE agent.

Background

A TSE strain is currently defined as an agent that has distinct and reproducible biochemical and molecular characteristics and which, when serially passaged through congenic or transgenic mouse lines, produces consistent characteristics of relative incubation period, spongiform lesion profile, molecular profile, and immunopathology.

BSE is defined as a TSE disease of cattle. The apparent consistency of the disease leads to the suggestion that the BSE epidemic was caused by a single strain of agent, although the existence of two additional types (defined as H-BSE and L-BSE) has been recognised.

Since the discovery of these BSE types, which are collectively also referred to as atypical BSE, the BSE agent that is responsible for the BSE epidemic is now referred to as classical BSE (C-BSE). The term BSE is used to include all three forms.

Based upon primary passage in RIII mice, strain typing suggests that a single strain is responsible for the majority of suspect cases of BSE, classical or atypical, and vCJD tested so far. However, transgenic (Tg) mice are increasing being used to elucidate the biological properties of individual isolates. The most widely published and best-characterised models relevant to the characterisation of small ruminant isolates include Tg338 mice (which overexpress the ovine VRQ gene) and Tg110 mice (which overexpress the bovine gene) amongst others. Transgenic lines such as Tg501 and TgShpXI which overexpress the ovine ARQ transgene have also been employed.

Biochemical features cannot at present be equated with the definition of a strain, but only as characteristics of a TSE type or strain. They do, however, serve as a very useful initial screen to identify isolates which merit further investigation. Certain biochemical properties of PrP^{res} as seen in Western blots (in particular the glycoform ratio, the molecular weight of the unglycosylated band, and the intensity of labelling with antibodies directed at the N terminal of the PrP) correlate well with the BSE diseases, novel TSEs and vCJD, and with the agent as isolated in mice.

Whilst there may be a large number of TSE strains in small ruminants, current focus is on distinguishing the C-BSE agent from all others, on the basis that only the C-BSE agent is established as zoonotic.

The basis for the zoonotic property of the C-BSE agent is not understood. It could be a result of:

- an intrinsic property of the agent
- passage through cattle and an intrinsic property of bovine prions
- simply a consequence of dose related to consumption of beef products compared to sheep products

In the absence of evidence to the contrary, it is generally assumed at present that zoonotic potential is an intrinsic property of the C-BSE agent. How this relates to any of the currently known and measurable properties of the agent is unknown. Therefore, in attempting to define the C-BSE agent, all that can be said at present is that it shows the following characteristics in all cases tested:

- low molecular weight of the unglycosylated band of PrP^{res} on Western blot (WB)
- type 4 glycosylation profile (i.e. PrP^{res} glycoprofile with a dominating diglycosyl moiety) on WB
- poor or absent binding of antibodies directed at N-terminal epitopes (ovPrP 93-97) in WB or ELISA formats under certain conditions
- biological phenotype characteristics (e.g. IHC and vacuolation distribution, WB, PrP^{Sc} distribution patterns in the brain revealed by IHC, relative incubation periods), which are indistinguishable from those of known C-BSE controls, in mouse bioassay

These characteristics have been found to be 100% consistent for the C-BSE agent.

However, on current evidence none of these characteristics, either alone or in combination, can be described as exclusive to the BSE agent.

Congruity with all of the above criteria will be interpreted as ‘the agent in question cannot be distinguished from the BSE agent’.

Variation of one or more characteristics will be interpreted as ‘the agent in question is not identical to the BSE agent’.

This manual contains the detailed protocols for biochemical discriminatory methods to enable discriminatory testing to be undertaken.

2. Testing strategies

Animal selection

Animals are currently selected for TSE testing through passive or active surveillance of the sheep and goat populations.

Sample requirements

The minimum sampling requirement for any animal from either source population is the brainstem (at the level of the obex) and part of the cerebellum, both of which can be accessed through the foramen magnum using a proprietary sampling spoon (See sampling guidance document: 'Sample requirements for TSE testing and confirmation – guidelines', for full details of sampling requirements and methods).

In summary, the quantity of tissue taken for testing should be sufficient to provide the following:

- a hemisection of fresh brainstem at the level of the obex, for the initial rapid test
- a fixed cross-section, or hemi-section of brainstem at the level of the obex for confirmatory IHC
- sufficient remaining fresh-frozen medullary tissue (adjacent to the obex) for primary molecular testing (discriminatory WB) and possibly a range of secondary and tertiary testing (5g to10 g whenever possible)
- brain tissue from other areas in addition to the brainstem is always an advantage, as are lymphoreticular tissues when available

Although not essential for statutory purposes, brain tissue from areas other than the brainstem will be important in characterising the scrapie strain present if results differ from the expected confirmation at the level of the obex.

Initial TSE monitoring testing

Screening testing

Active surveillance samples are subjected to one of the current commercial TSE rapid tests. It is a requirement that this initial monitoring must be capable of detecting atypical scrapie.

Passive surveillance samples from clinical suspects may bypass rapid test and proceed directly to confirmatory testing.

Confirmatory testing

Any animal found positive by any initial test, in either the active or passive surveillance programmes, shall be subject to confirmatory testing by one or more of the following methods – immunohistochemistry (IHC), Western blot (WB), or histopathological examination.

Any TSE case which is confirmed, except atypical scrapie cases, should be subject to primary molecular testing to determine whether it is classical scrapie, or BSE-like.

Primary molecular testing with a discriminatory immuno-blotting method

Any positive, non-atypical (Nor98) scrapie, TSE isolate confirmed in sheep or goats pursuant to initial screening will be subjected to discriminatory WB using one of the protocols listed in part 4 of this Handbook. These tests were originally assessed and approved, using a ring trial coordinated by the EURL STEG, which confirmed the ability of these tests to discriminate between C-BSE (either ovine or bovine) and classical scrapie (ovine).

Possible outcomes of the primary molecular testing

If the results of the primary molecular testing (discriminatory WB) are compatible with the defined characteristics of classical scrapie isolates (see below for interpretational criteria within the individual method protocols), the case is confirmed as classical scrapie positive, and no further testing is required.

If the results of the primary molecular testing (discriminatory WB) are inconclusive or different from above and cannot exclude BSE, then secondary molecular testing with additional molecular testing methods must be pursued.

A poor-quality WB (e.g. one that is faint, or overloaded) that does not enable clear identification of three bands (diglycosylated, monoglycosylated and unglycosylated) cannot be interpreted and must be repeated.

Full details of test interpretation and specific criteria for referral can be found within the protocols for each method in the following section of this Handbook. Broadly, cases that require referral include those with one or more of the following characteristics:

- evidence of a low molecular mass for the unglycosylated band of PrP^{res}, relative to scrapie, and, if available, C-BSE controls. (This excludes those isolates with the very low 12-14 kD band known to be associated with atypical/Nor98 scrapie)
- detectable reduction in immunoreactivity with the N-terminal specific antibody (binding to the epitope ovPrP 93-97) when compared with the primary antibody (binding to the core region of PrP)

This step can be more informative in samples with abundant PrP^{res} if they are subjected to a high PK concentration. However, samples with weak core-antibody signal cannot be subjected to this treatment as the aggressive PK digestion may obliterate all PrP^{res}.

Secondary molecular testing with additional molecular testing methods

If the Western blot results are still unresolved and fixed sample is available, investigate further on a case-by-case basis using some or all of the following methods:

- a repeat WB using a different method/antibodies raised to different epitopes
- discriminatory IHC

If the outcome of the testing indicates that the isolate is indistinguishable from C-BSE or inconclusive with regard to C-BSE status it will go forward for:

- discriminatory PMCA, followed by
- mouse bioassay

Tertiary testing strategy – mouse bioassay

For resolving small ruminant TSE cases each isolate will be bioassayed in different mouse lines, e.g. tg338, tgShpXI and tg110 mice that express the ovine and bovine prion protein gene.

3. Reporting and referral systems

Data collection and provision

All cases reported should be accompanied by a breakdown of all the data requested.

For cases being referred for secondary testing, the accompanying data should be broken down as indicated in the tabulation attached (appendix 2). This tabulation enables:

- provision of a standardised data summary for each referred isolate, regardless of where the testing is performed
- the collection and direct comparison of similar, objective data on each individual case and the retrospective reinterpretation / regrouping of any individual parameter if required

It will assist with the classification, definition and preliminary epidemiological assessment of any previously unrecognised isolates that may arise in future.

How to refer an isolate

Each case referred to the TSE WOAH RL or TSEglobalNet should be notified in advance by email, to the UK NRL mailbox: uk.tse.nrl@apha.gov.uk and the TSEGlobalNet mailbox: TSE.GlobalNet@apha.gov.uk including a copy of the referral form (see appendix 3). It is particularly important that this notification includes an indication of the amount and quality of tissue available for subsequent testing.

Please supply all available tissue from each case.

All movement of material must be covered by the relevant export and import permits. A copy of the current APHA import permissions can be obtained by request via the TSEglobalNET mailbox. Copies of any additional relevant documentation will be provided by the TSE WOAH RL/UK TSE NRL as required.

Please quote all relevant licence numbers on all paperwork.

Packages should be compliant with International Air Transport Association (IATA) regulations.

Packages should be addressed as follows:

For the attention of TSE WOAH RL/UK TSE NRL
Diagnostic Team
Building 96
APHA Weybridge
Woodham Lane
New Haw
Addlestone
Surrey
KT15 3NB
UK

How results will be reported

The formal reporting of the final results of secondary testing will be the responsibility of the competent authority in the referring country.

Note: In accordance with Resolution No. XXVIII, adopted by the WOAH International Committee in May 2004, WOAH Reference Laboratories (of which APHA is one) are now required by mandate to inform the WOAH Central Bureau of confirmed positive diagnostic results for diseases that are reportable to the WOAH. Such information will be sent by the

WOAH Central Bureau to the WOAH Delegate of the country concerned before any publication.

4. Discriminatory Western blotting methods

The APHA Bio-Rad TeSeE-based hybrid Western blot method

The Bio-Rad TeSeE Western blot assay is a commercially available test kit that allows for the detection of PrP^{res} in nervous or peripheral tissues collected from infected animals which can be distinguished from normal PrP, both by its protease resistance and molecular size. This abnormal protein is a unique identifier of Transmissible Spongiform Encephalopathy (TSE). This test has been developed as a confirmatory test of initial reactive samples identified by rapid screening tests and uses single antibody detection.

The assay procedure includes the following steps: Sample homogenisation, digestion of PrP^{sen} with Proteinase K, purification, and concentration of PrP^{res}, electrophoretic migration of the protein and transfer onto a membrane, immunoblotting, and detection of signal.

Here, this confirmatory test is used with a double antibody detection method which enables discrimination to be made between cases of natural bovine BSE, natural ovine scrapie, and experimental BSE and natural scrapie in sheep.

Safety

Work with prions or potentially prion-containing materials has to be performed in strict accordance with National Safety Regulations.

Laboratories MUST adhere to National Safety Regulations, but the following information, published by The Advisory Committee for Dangerous Pathogens (ACDP) is available for guidance: ‘Transmissible Spongiform Encephalopathies (TSE) agents: safe working and the prevention of infection’. Copies can be obtained (ISBN 0113221665), from Department of Health (London) Stationery Office (Telephone +44 (20) 7873 9090). An update is available on the UK Department of Health web site (Minimise transmission risk of CJD and vCJD in healthcare setting).

Materials

Chemicals and reagents

This Western immunoblotting protocol uses chemicals and reagents supplied with the TeSeE™ Universal Western Blot Kit (refer to www.bio-rad.com for distributors) and from other suppliers. The shelf life of all kit components is 1 year after production date if stored at + 5±3°C. For actual expiry date see kit or components label.

TeSeE Universal Western Blot kit

Bio-Rad Tel No: +44 (0) 17585 812121

Cat No: 355 1169. Store at +5±3°C

Kit includes:

Grinding tubes (containing ceramic beads in a buffer solution)

A Denaturing Solution

B Clarifying Solution

PK Proteinase K

Ab I Primary antibody: anti PrP monoclonal antibody (10x)

Ab II Secondary antibody: Goat anti-mouse IgG (H+L)-HRP (10x)

Bl Blocking solution (10x)

Additional reagents

Reagent	Supplier
Laemmlli Sample buffer	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0737 Store at 18°C to 30°C
2-Mercaptoethanol	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0710 Store at 18°C to 30°C
SDS	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0301 Store at 18°C to 30°C
Criterion XT 12% Bis-Tris	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 345 0118 Store at 18°C to 30°C

XT-MOPS x20	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0788 Store at 18°C to 30°C
Ethanol (Normapur)	VWR – Tel No: +44 (0) 1455 558600 Cat No: 20821-296 Store at 18°C to 30°C
Tris/CAPS x10	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 161 0778 Store at 18°C to 30°C
Tween 20	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 6531 Store at 18°C to 30°C
PBS x10	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 355 1169 Store 18°C to 30°C
ECL Substrate	Amersham - Tel No: +49 (0) 761 451 90 Cat No: RPN2109 Store at +5±3°C
Goat Anti-Mouse HRP	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 172-1011 Store at -20°C or lower
Non-fat milk blocking reagent	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No 170-6404 Store at 18°C to 30°C
Primary Antibody RIDA® mAbP4	R-Biopharm Rhone LTD -Tel No: +44 (0) 141 9452924 Product No: R8007 Store at +5±3°C
Sigma Biotinylated Molecular Mass Standards	Sigma-Aldrich -Tel No: +44 (0) 800 717181 Product No. B2787. To contain standards between approximately 10-200kDa. Diluted in SDS Sample buffer. Store at -20°C or lower
Streptavidin - HRP	Sigma-Aldrich - Tel No: +44 (0) 800 717181 Cat No. S2438 Store at -20°C or lower

****For X-ray development only**

Reagent	Supplier
**X-ray film developer	Kodak - Tel No: +44 (208) 3189441 Developer LX24 Product No 507 0933 Store at 18°C to 30°C
**X-ray film fixer	Kodak - Tel No: +44 (208) 3189441 -Fixer AL4 Product No 507 1071 Store at 18°C to 30°C
**X-ray cassette	To fit x-ray film
**X-ray film	Amersham Tel No: +49 (0) 761 451 90 ECL RPN2103K or equivalent Store at 18°C to 30°C

Equipment

Equipment	Supplier
**Development folders	Applied Biosystems Tel No: +44 (0) 800 515840 Cat no: T2258
1ml Calibration syringes	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 355 1174
2ml Micro test tubes with safe lock caps	Eppendorf - Tel No: +44 (0) 7884263628 Cat No: 0030 120.086
Graduated pipettes	5, 10, 25ml
PVDF membrane (0.2 μm pore size)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 162 0175
Filter paper	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 4085
Cotton buds	

TeSeE PRECESS 48	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 359 0200
Heating Block (to fit 2ml Eppendorf tubes)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 358 9046
Centrifuge (capable of 15 000g)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 358 9190
Criterion XT Cell (Includes Roller)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 165 6001
PowerPac HC power supply	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 165 5052
Criterion XT Blotter (Includes 2 sandwich cassettes and 4 sponges)	Bio-Rad - Tel No: +44 (0) 17585 812121 Cat No: 170 4070
Balance	With draft shield. Capable of weighing 0.4g ±0.5mg
Balance	Capable of weighing 310g ±0.01g
Biological Safety Cabinets	Class I (within a containment laboratory meeting national requirements for handling TSEs)
Freezer	Capable of being controlled at –20°C or lower
Freezer	Capable of being controlled at –70°C or lower
Imaging machine and PC (if detecting results with imager)	Bio-Rad - Tel No: +44 (0) 17585 812121 Fluor-S MultiImager or Versa Doc Model 1000
Magnetic Stirrer	Capable of maintaining 200rpm
Membrane incubation boxes	To fit the area of PVDF membrane used
Pasteur pipette	Plastic, disposable

Photographic trays (if detecting results by x-ray developing)	Three, to fit x-ray film
Pipette	5-40µl (Accuracy 4%, Precision 2.5%)
Pipette	40-200µl (2% Accuracy, 2% precision)
Pipette	200-1000µl (Accuracy 2%, Precision 2%)
Refrigerator	Capable of maintaining +5±3°C
Rocking Platform	Bibby Sterilin Ltd - Tel No: +44 (0) 1443 830830 Product No: Gyro-Rocker STR9
Tray	To accommodate transfer sandwich assembly
Vortex mixer	Thermo Instruments - Tel No: +44 (0) 870 609 9203 Product Code: Fuson Whirlimixer

Protocol

Planning

A positive bovine classical BSE and positive classical ovine scrapie are always included on each run to validate the processing. Molecular mass markers are always included to allow for analysis of the banding patterns and molecular mass determination. The number of samples to be processed will vary on each run depending on the submissions and additional controls may be added if the samples are unusual and further examination required. The gel plan will be prepared to include all samples to be examined and relevant controls required.

Each gel is duplicated so that matching membranes will be produced to probe with different antibodies. This enables a comparison for each sample between its affinities for two antibodies targeted at different epitopes of the prion protein.

An example of a gel plan is shown in Fig 2.

Sample purification

(Note: This stage is carried out in an appropriate safety cabinet according to National Safety Regulations.)

Sampling and homogenisation

Dissect and deposit 350mg ± 40mg of brain tissue in a grinding tube.

Grind the sample in the Ribolyser for 45 sec at a speed of 6.5 for neural tissue.

Mix by inversion. Aspirate 500l with a calibration syringe.

Transfer each 500µl into a 2ml micro-test tube.

Sample digestion

Distribute 500l of reconstituted proteinase K solution (see kit insert for method) into each micro-test tube and mix thoroughly by inversion (10 times).

Incubate at 37°C ± 2°C for 10 mins.

Precipitation of PrP^{Sc} and concentration

Distribute 500l of reagent B into each tube and mix thoroughly by inversion (10 times)

Centrifuge at 15 000g at 20°C for 7 minutes.

Sample clarifying

Discard the supernatant and dry the tube for 5 mins by inversion.

(Note: Use a disposable cotton bud to remove any excess liquid from the tube and lid, taking care not to disturb the pellet).

Distribute 100l of Laemmlli solution (see kit insert for method) and incubate for 5 mins at room temperature (18°C to 30°C).

Completely resolubilise the pellet by aspiration/dispensing with a pipette.

Incubate at 100°C ± 5°C for 5 minutes, then homogenise by vortexing.

Centrifuge the tubes for 15 mins at 15,000g at 20°C.

Transfer the supernatant into a new micro-test tube. The procedure can be split over 2 processing days at this point and the denatured sample frozen at -20°C for 24 hours. The samples should be thawed prior to use.

Protein separation

(Note: This stage is carried out in a safety cabinet.)

Gel preparation

Remove the plastic strip on the bottom of the plate and place the gels in the migration tank.

Pour the MOPS x 1 migration buffer (see kit insert for method) on each side of the gel up to the top of the wells and into the Criterion XT Cell electrophoresis migration tank. Remove the combs and rinse each well with migration buffer.

Heat the samples and known controls at 100°C ± 5°C for 4 minutes.

Immediately load 15l of each sample per well according to the gel plan.

Load 5µl of Sigma Biotinylated Marker diluted 1:4 in Laemmlli Buffer and heated at 37’C for 2 hours as per reagent instructions (or other markers according to their specific instructions) in the outer most wells each side.

Repeat the loading on another gel, to give a duplicate gel that is identically loaded and can be probed with a different antibody.

Run the gels at room temperature (18°C to 30°C) for 50 minutes at 200V.

Protein transfer

Protein transfer preparation

Cut the membrane to the gel dimensions. Label each membrane in the corner so they can be correctly identified at a later stage.

Immerse the membranes in pure ethanol for 15 seconds.

Rinse in distilled water for 5mins

Equilibrate in transfer buffer (see kit insert for method) for 10 minutes.

Gel sandwich preparation

Equilibrate the gel in transfer buffer for 10mins.

Pre-soak the fibre pads and filter paper in transfer buffer.

Respectively place on the red side of the cassette: fibre pad, filter paper, membrane, gel, filter paper, and fiber pad.

Transfer onto PVDF membrane

Fill the Criterion XT blotter to the indicated limit with approximately 1.5l of transfer buffer. Place an ice pad in the holder of the transfer tank and run with agitation using a magnetic stirrer for 60 mins at 115V.

Immunoblotting

Upon completion of transfer, open the blotting assembly and remove the membranes for development. Soak membranes in Wash solution 2 (see kit insert for method) then in pure Ethanol for 10 seconds. Rinse in distilled water for 5 mins.

Incubate the membrane to be detected with the kit antibody (SHA31) in 40ml of blocking solution (see kit insert for method) for 30 mins under medium agitation.

Incubate the membrane to be detected with P4 in 50ml of Bio-Rad non-fat milk blocking reagent blocking solution for 30mins under medium agitation (Make up a 5% solution with Wash solution 1 and dissolve for at least 1 hour before use).

Note: The non-kit Bio-Rad Non-fat milk blocking reagent is used for probing with any other primary antibodies. Make up a 5% solution with Wash solution 1 and dissolve for at least 1 hour before use.

Dilute the kit primary antibody (see kit insert for method)

Dilute the P4 primary antibody 1:5000 (1mg/ml stock solution).

(5µl mAb P4 in 25mls Wash solution1).

Eliminate the blocking solution from both membranes.

Incubate the first membrane in 30ml of diluted kit primary antibody SHA31 at 18°C to 30°C for 30 mins under medium agitation.

Incubate the second membrane in 25ml of diluted mAb P4 primary antibody at 18°C to 30°C for 60 mins under medium agitation.

To allow all membranes to be taken through the washing and detection stages together, the SHA31 membrane should be placed at +4°C for a further 30 mins whilst the P4 membrane continues to the end of the 60 mins incubation period.

Using Wash solution 1, briefly rinse then wash twice for respectively 5 and 10 mins under fast agitation (100ml per membrane and per cycle).

Dilute the kit secondary antibody for the SHA 31 membrane (see kit insert for method).

Dilute the secondary antibody for the P4 membrane, Bio-Rad Goat Anti-Mouse HRP 1:5000. (10µl in 50mls Wash solution1).

Note: If using Sigma Biotinylated Markers add Sigma HRP (dilution 1:30 000).

Incubate the membranes in the appropriate diluted secondary antibody at 18°C to 30°C for 20mins under medium agitation.

Using wash solution 1, briefly rinse then wash three times respectively for 5, 10 and 10mins under fast agitation (100ml per membrane and per cycle). Place the membrane in Wash solution 2 under medium agitation.

Prepare ECL Substrate reagent (see kit insert for method).

Eliminate the wash solution 2 and drain the membrane on absorbent paper without blotting. Place the dried membrane onto a plastic transparency and add the substrate solution. Cover with another transparency and distribute the solution evenly over the membrane eliminating excess and air bubbles. Incubate for 1 min.

Signal detection using a CCD imager

Blot away any excess liquid on the transparency before placing it into the Imager. Place the membrane into the Bio-Rad Multi-Imager and detect the light signal using 1- and 10-minute exposures.

The membrane should be exposed until a strong signal of the positive control is visible.

Expose longer or shorter times for optimal signal visualisation.

Label the blot with sample and control details from gel loading plan and also include run number, method of processing, antibody and detection time, and operator initials and date.

Signal detection using X-ray film

Place the development folder in the X-ray cassette and turn off the safelight.

Place a sheet of S-ray film on top of the membrane and close the cassette. Expose the membrane to the X-ray film. The actual exposure time will vary according to the samples processed and the suggested time is . Expose the film longer or shorter times to achieve optimal signal visualisation.

Place the film in a tray of x-ray developer solution for 45 seconds with occasional agitation.

Wash the film for 1 minute in a tray of tap water

Place the film in X-ray fixer until the film becomes transparent.

Thoroughly wash the film in distilled water and then hang the film up to dry.

Interpretation of the APHA bio-rad based-hybrid Western immunoblot results

This method is capable of identifying PrP^{Sc} profiles associated with classical bovine BSE, atypical H and L-type bovine BSE, classical ovine scrapie, atypical ovine scrapie, and experimental BSE in sheep.

Using this Western blot method with monoclonal antibodies SHA31 and P4, clear but subtle differences in the molecular weight position and antibody affinity are observed between the different PrP^{Sc} profiles described in the summary. A representative image of each type is shown below in figures 1a to 1d.

A negative sample will have been totally digested by proteinase K, and no signal will be visualised with either antibody, illustrated in figure 1b (lane 4).

Any samples that give a banding profile that is not consistent with either classical or atypical scrapie should be referred to the TSE WOAH RL/UK TSE NRL for a second opinion and further investigation where necessary.

Limitations of the test

The method requires sufficient tissue from the optimal brain area, in order to maximize the amount of PrP^{Sc} loaded from each sample.

If the quantities of brain material are too low (a situation we have encountered with active surveillance), the number of Western blots that could be run from a single sample may not be sufficient to obtain an accurate interpretation and there may not be enough PrP^{Sc} in underweight samples to confirm a diagnosis even though the sample may be positive.

It is important to compare the molecular weights of samples, relative to each other and the controls on the same gel.

Care must be taken in interpretation, as samples that have not been digested correctly may initially appear positive. These should be repeated.

Strong positive samples may give a saturated signal intensity which will obscure visual interpretation of the banding profile. This type of sample requires repeat immunoblotting following dilution, so a clear banding pattern is observed.

The full banding profile may not be visualised in weaker samples or where the optimal tissue for atypical scrapie has not been submitted.

Atypical TSE's differ in their PrP^{Sc} distribution and so the correct brain area is critical for clear, accurate results e.g. medulla for classical scrapie and cerebellum for atypical scrapie.

We have shown that differences in the ovine genotype do not appear to give any variation in the banding obtained for scrapie samples, but autolysed samples can run quicker through the gel and may resulting in a lower molecular weight than the equivalent fresh sample. At present we do not know whether mAb P4 results are affected by autolysis.

We have only assessed certain genotypes of experimental sheep-BSE and some molecular weight variability of natural sheep-BSE, if it is in the ovine population, cannot be ruled out at this stage.

Result summary

Classical Bovine BSE

Signal and low molecular mass migration (compared to ovine) with mAb SHA31.

No signal with mAb P4.

Predominance of diglycosylated band.

(See figures 1a to 1d)

Classical Ovine scrapie

Visible signal and higher molecular mass migration (compared to bovines) with mAb SHA31.

Visible signal with mAb P4.

(See figures 1a to 1d)

Unusual Bovine BSE

Potential ‘H’ Type

Visible signal and high molecular mass migration (compared to bovine control) with mAb SHA31.

Equivalent signal with mAb P4.

Predominance of diglycosylated band.

(See figure 1a)

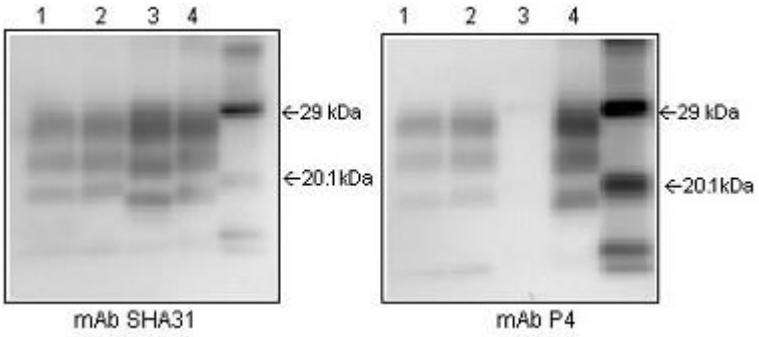


Figure 1a

Key code

1 = Atypical BSE (H type)

2 = Atypical BSE (H type)

3 = Classical BSE

4 = Classical scrapie

Potential 'L' Type

Visible signal with mAb SHA31.

Molecular mass migration can be equivalent or lower than bovine control with mAb SHA31

No signal with mAb P4.

Reduction in the intensity of the di-glycosylated band compared to the monoglycosylated band. This may give the appearance of equal intensity of both bands or a predominance of the mono-glycosylated band.

NB The glycoprofile can be difficult to determine if the signal is saturated, therefore dilution of the sample may be necessary.

(See figure 1b)

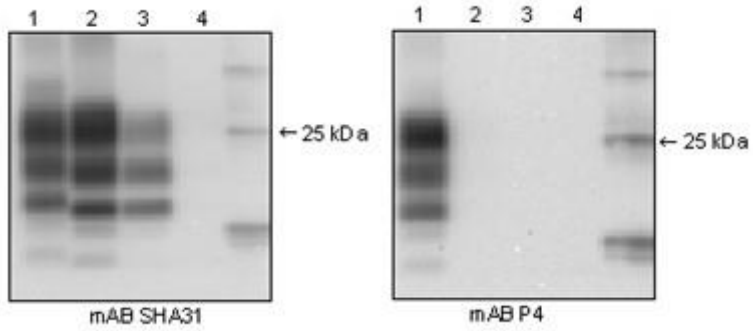


Figure 1b

Key code

1 = Classical scrapie

2 = Classical BSE

3 = Atypical BSE (L type)

4 = BSE -ve

Atypical Ovine Scrapie

A multiple band profile is seen with mAb SHA31 and mAb P4.

The bands show a significant downwards shift compared to the classical scrapie control with the fourth band being lower than 15kD (typically around 12kD).

(See figure 1c)

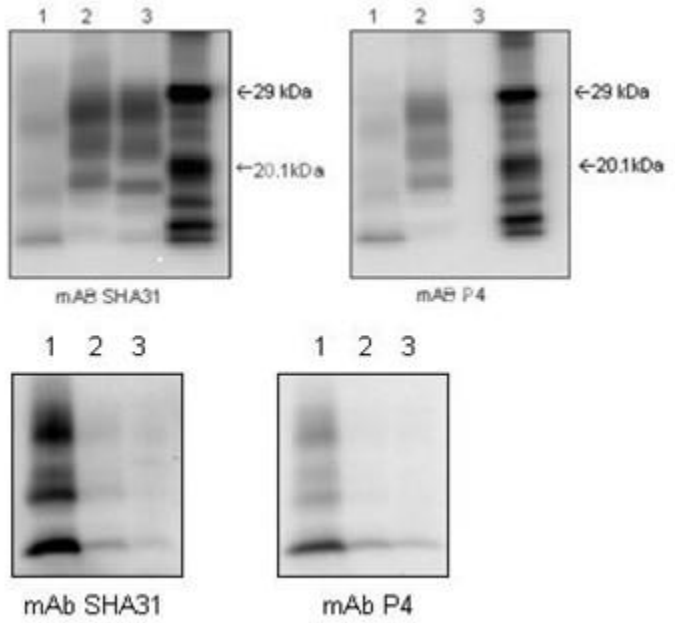


Figure 1c

Key code

1 = Atypical scrapie

2 = Classical scrapie

3 = BSE

1 = Atypical scrapie Neat

2 = Atypical scrapie (1:10)

3 = Atypical scrapie (1:50)

Experimental BSE in sheep

Strong signal and lower molecular mass migration (compared to ovine scrapie) with mAb SHA31.

Much reduced signal, or no signal with mAb P4.

(See figure 1d)

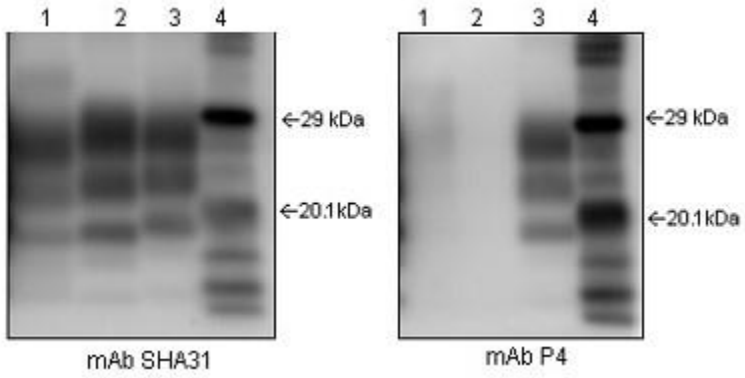


Figure 1d

Key code

1 = BSE in sheep

2 = Bovine BSE +ve

3 = Ovine scrapie +ve

4 = Biotinylated marker

Negative

No signal with either mAb SHA31 or P4

Suggested gel plan

Figure 2: suggested gel plan showing placement of end biotin markers, samples 1 to 14, controls: Bovine BSE positive, Ovine Scrapie positive

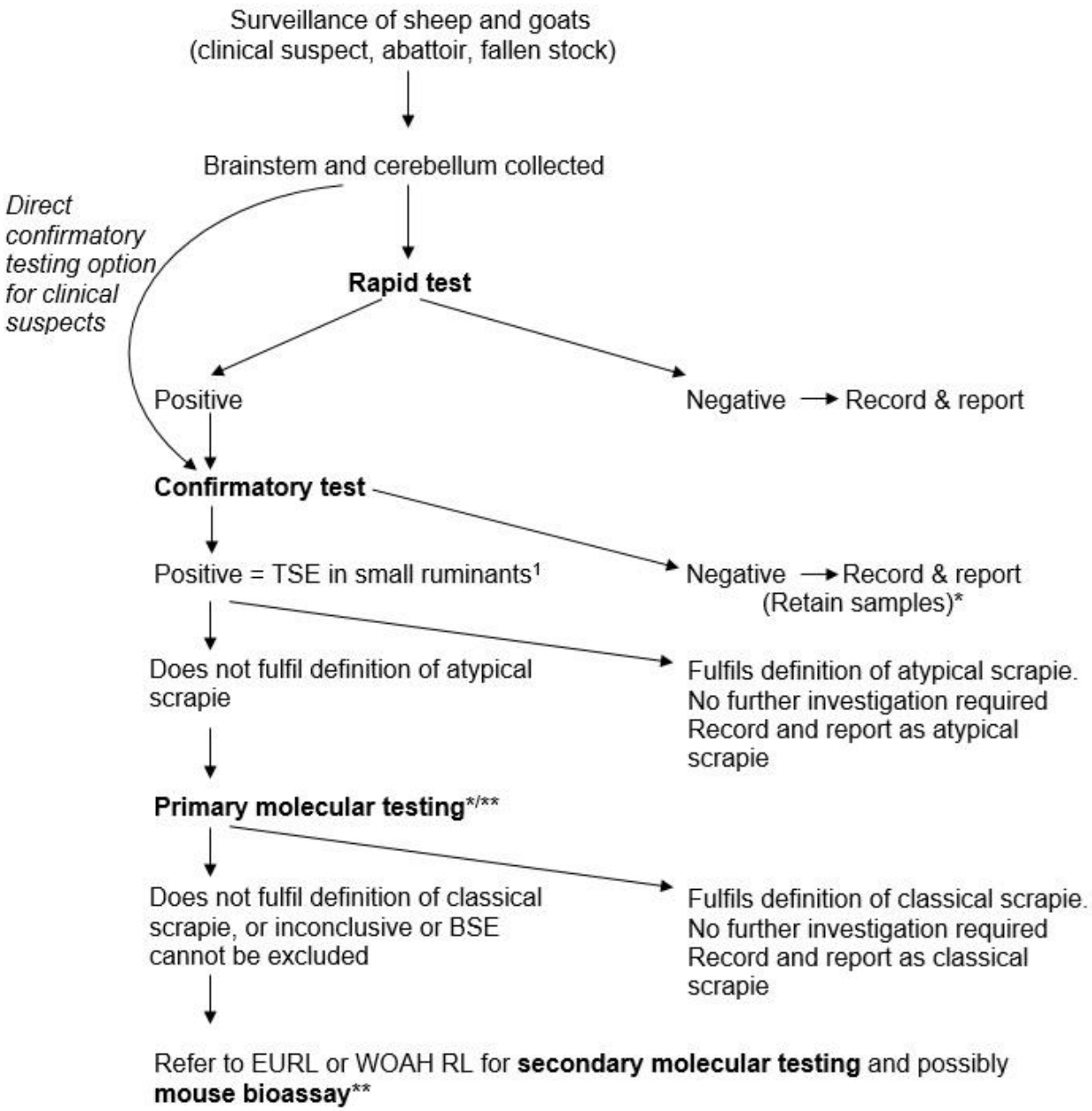
Biotin marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Bov BSE +ve	Ov Scrapie +ve	Biotin marker

References

Manufacturers instructions – included in Bio-Rad TeSeE Western blot kit.

5. Appendices

Appendix 1: testing strategy flowchart



*These steps are conducted by the submitting laboratory

** All discriminatory testing to be QA'ed by TSE WOAH RL/UK TSE NRL

[1]Current criteria for definition can be found in
http://www.efsa.europa.eu/en/efsajournal/pub/276.htm

Appendix 2: Referral format for SR TSE positive isolates

The following information should be recorded.

Table: Referral format for SR TSE positive isolates to the TSE WOAH RL/ TSE UK NRL for discriminatory testing

Animal ID	
Age	
Genotype	
Breed	
Rapid test used - result	
Confirmatory test used	
- overall result	
(Ab used for IHC)	
If IHC unusual (i.e. different neuroanatomical distribution, or morphological type *see website/ guidelines)*	
Ab used	
Distribution of immunoreactivity (i.e. present/absent)	
DNV?	
V?	
Sol tract?	
Other?	
Discriminatory WB:	
Method used	
MW of unglycosylated band	
Relative staining intensity: PrP N-terminus specific < = or > PrP-core specific antibody).	

Amount and type of material remaining from this case?	
SRT number (issued by TSE WOAH RL/TSE UK NRL)	

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