TSE EURL

Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta - Turin

Istituto Superiore di Sanità - Rome

APPROACH FOR THE PROVISIONAL CLASSIFICATION OF BOVINE TSE ISOLATES

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The examination by histopathology, immunohistochemistry, Western blotting and bioassay of bovine isolates from individuals with clinical signs throughout the BSE epidemic has supported the hypothesis that the epidemic has been sustained by a single type, or strain, of BSE. However, the development of sensitive PrP^{res} immuno-detection diagnostic techniques and their application through active surveillance in non-suspect populations have led to the detection of a small number of geographically widespread sporadic cases of deviant types, predominantly in older animals. These isolates have now been confirmed in mice as distinct strains, and have been operationally defined as H- (high) or L- (low) type based on the molecular mass of the unglycosylated fragment of PK resistant PrP in Western blot, as opposed to the classical form of BSE (C-type BSE).

L-type behaves in Western blots like the cases initially identified in Italy (initially described as BASE; bovine amyloidotic spongiform encephalopathy). For the time being L-type and BASE are considered to be the same.

It is now a regulatory requirement for all positive bovine isolates to be classified by discriminatory methods in L-type, H-type or C-type based on distinctive molecular characteristics of PK resistant PrP. This discriminatory test shall be performed by a laboratory, appointed by the competent authority, which has participated successfully in the latest proficiency testing organised by the EU reference laboratory for discriminatory testing of confirmed BSE cases.

The following blotting guide has been prepared on behalf of the European Union Reference Laboratory (EURL) for the TSE Strain Typing Expert Group by Prof. Jan Langeveld, Lelystad, based on the 2007 publication by Jacobs et al.

In order to have confidence in the results of such a test, it is vital that the appropriate controls should be run on the same gel as the suspect sample. Appropriate controls would include samples previously confirmed as C, H and L type BSE, either in the laboratory of origin, or through referral of the sample to a laboratory with the correct control materials available.

Please note: A protocol for the discriminatory testing of positive BSE samples (The APHA Bio-Rad TeSeE-based Hybrid Western blotting Method) is made available to NRLs in the EU reference laboratory guidelines on discriminatory testing and classification — 'TSE strain characterization in small ruminants: A technical handbook for National Reference Laboratories in the EU'.

Please note: If you suspect that you have an unusual sample that requires further characterization, it is recommended that you contact the EURL (eurl.tse@izsto.it) to discuss the options available to you.

A 2-blot protocol for PrP^{res} typing in BSE from cattle.

Introduction

The initial classification of C-type, H-type and L-type BSE using different parameters (as summarized in table I) has been undertaken by the European funded NeuroPrion network of excellence. The following parameters are used:

1. molecular migration of PrP^{res} bands
2. differential binding to PrP-specific antibodies (to this purpose antibody groups A, B and C have been defined, see Table II)
3. PrP^{res} glycoprofiles
4. susceptibility to proteinase K (PK)
5. number of non-glycosylated PrP^{res} bands

Table I: Discrimination between BSE-types based on molecular properties of PrP^{res} (from: Jacobs et al., 2007)

BSE type	size difference [a] in kDa	binding to 12B2	glycoprofile[b] di-glyc (%)	deglycosylation with PNGase F[c] (163-242 epitopes)	proteolytic susceptibility pH 8/pH 6.5[d]
C	ref	no	>50	1 band	> 0.7
H	+1.4	yes	dual character[e]	2 bands	< 0.6
L	-0.3	no	<50	1 band	< 0.6

[a] Approximate difference value with C-type for the nonglycosylated band of the PrP^{res} population in 17-19 kDa region; tested with group B antibodies 9A2, L42 or 6H4.

[b] Percentages of diglycosylated fraction should be compared together with C-type.

[c] Two bands can only be observed with group C antibodies like 94B4 and SAF84 that bind to the C-terminal domain 163-242 of bovine PrP.

[d] Ratio calculation, see Jacobs et al., 2007.

[e] Depending on the use of antibodies of groups A & B or of group C (see Table II).

Table II: gr ouping of antibodies for recognition of bovine BSE-t ypes updated from Biacabe et al., 2007

antibody group	antibody	region of binding in boPrP
group A	SAF32; 4F2; 12B2**; P4	62-107
group B	9A2; RB1; 12F10[a]; 6C2; F89/160.1.5; Bar233; L42**; Sha31**; 6H4	108-157
group C	SAF70; SAF60; SAF84**; 94B4**, F99/97.6; R524	157-242

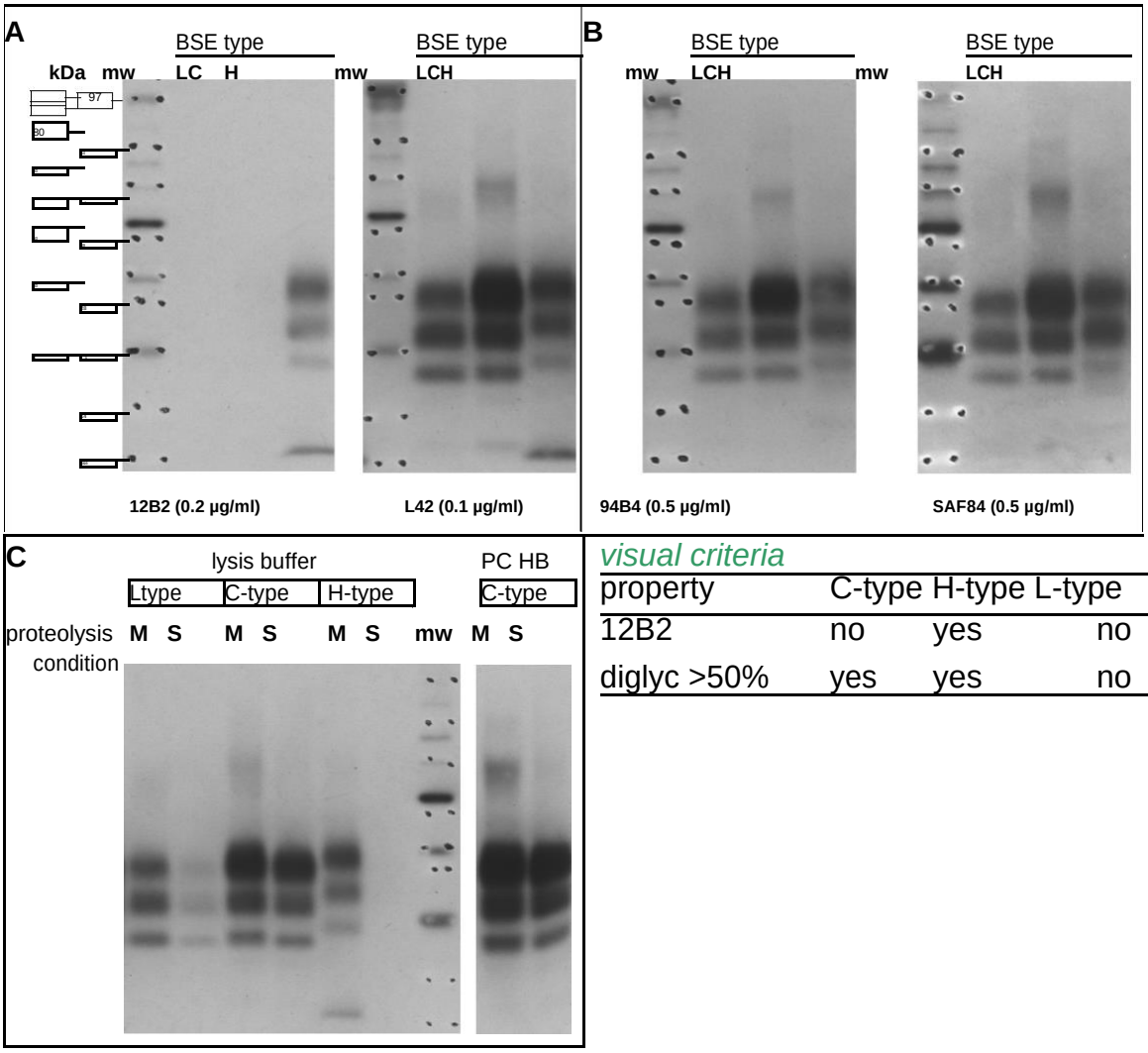
** Antibodies marked with asterisks are the preferred antibodies for protocols A-D because of high affinity.

For standard discrimination of these three types only 2 parameters are required, and can be performed by visual inspection using antibodies of sufficiently high affinity to detect PrP^{res} (Fig. 1A):

1. Binding to PrP^{res} *N* - terminus specific antibody 12B2 (a group A antibody) compared to L42 (a group B antibody). Migration position of PrP^{res} bands of H-type is higher up than that of C- and L-type, due to the *N* - terminal epitope of 12B2 which is retained during digestion with PK in substantial amounts only in H-type (Fig. 1A).
2. Glycoprofile differences between L-type on the one hand, and C- and H-type on the other hand using L42 (group B antibody) (Fig. 1A).
3. This double blot test of Fig. 1A yields sufficient criteria for discrimination of three types (see table "visual criteria").

4. Using a group C antibody like 94B4 or SAF84, the PrP^{res} glycoprofile of H-type cases is basically different from the profile obtained with a group A or B antibody (compare H-type lanes between Fig. 1B and 1A).
5. The susceptibility of C-type for proteinase K is nearly the same between mild or stringent digestion condition, while on the contrary PrP^{res} of L-type and H-type hardly survives the stringent condition (Fig. 1C). This is the case for homogenates prepared in different homogenisation buffers like lysis buffer and a homogenisation buffer from a commercial test kit for bovine BSE detection (Prionics®-Check Western) (see lanes "PC HB", Fig. 1C).

Figure 1



visual criteria

property	C-type	H-type	L-type
12B2	no	yes	no
diglyc >50%	yes	yes	no

Fig. 1: Features of BSE types. **A**, H-type reacts in nearly equal intensity to antibody 12B2 and L42, while C-type largely and L-type completely are devoid of the 12B2 epitope due to *N* - terminal cleavage of amino acids 101-104 by PK under standard digesting conditions (pH7.2, PK 50 µg/ml, 37ºC, 60 min, in lysis buffer). Also, a 7 kDa band is present (L42 panel) which is most prominent in H-type. The glycoprofile of the L-type is clearly different from C- and H-type, because in L-type diglycosyl fraction is about 50% or less of the total PrP^{res} signal, while in C-type and H-type it is the diglycosylated band represents the predominant fraction. **B**, the glycoprofile of H-type is dependent of the antibody used. With group C antibodies it exhibits a similar intensity of bands running at a position of di- and mono-glycosylated PrP^{res} while on the contrary with group A or B antibodies the diglycosyl band represents the most intense band (cfr. Fig. 1B with Fig. 1A). **C**, Further evidence for difference of H-type and L-type from C-type comes from using two digestion conditions. Antibody used: group B antibody L42 at 0.1 µg/ml. Only C-type resists in large a stringent PK digestion (S) compared to mild digestion (M). Applied tissue equivalents, 0.5 mg per lane. After digestion samples were first precipitated with 1-propanol. Lane mw, molecular weight markers Magic Mark™ XP and SeeBlue® Plus2 (Invitrogen). Geltype: 12% Bis/Tris NuPAGE using MOPS running buffer (Invitrogen). Developed with CDP*, film exposure 1 min. S = stringent digestion at pH8, ~1 µg PK/100µg wet tissue; M = mild digestion at pH6.5, ~0.1 µg PK/100 µg wet tissue.

Protocols:
These protocols are based on the report by Jacobs et al., 2007.

Solutions/materials:

water	distilled or reversed osmosis grade water
PBS	8 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, 0.38 g KH2PO4, water to 1 L. pH=7.2 (10.8 mM Pi, 138 mM NaCl, 2.7 mM KCl)
lysis buffer	0.5% (w/v) TritonX-100, 0.5% (w/v) sodium-deoxycholate, in PBS
1M CaCl2	1.47 g CaCl2.2H2O, dissolve in water; final volume 10 ml
4M HCl	add 5 ml HCl concentrated to 10 ml of water; final volume 15 ml
Tris/Ca	0.61g Tris, dissolve in 100ml water, bring at pH8.0 with ~1ml 4M HCl, add 100µl 1M CaCl2 → 1mM Ca in 50mM Tris pH8.0

Adjustment buffers:

pH6.5 buffer 200mM PO4 pH6.5	prepared as follows **A,** dissolve 13.8g NaH2PO4·1H20 in 500ml water; **B,** dissolve 14.2g Na2HPO4 in 500ml water; titrate 3 volumes of **A** with ~1-2 volumes of **B** till pH6.5.
pH8 buffer	200mM Tris+HCl pH8.0 prepared as follows. Dissolve 12.1g Tris in ~450ml water. Titrate to pH8.0 with ~11.5ml of 4M HCl. Make up final volume to 500ml with water.
PK	proteinase K (30 mAnson-U/mg lyophilisate, Merck 1.24568)
500 µl vial	500 µl Eppendorf safelock vial
thermostat	incubator with heating blocks with tight fitting for 500 µl vials
centrifuge	table top microtube centrifuge such as Heraeus Biofuge Pico
PK-stock	11mg PK in 1ml Tris/Ca (aliquot 50µl portions and store at -20 C; retains activity for at least 12 mo)
PK-fresh	on day of use prepare by diluting 50 µl PK-stock with 950 µl PBS → 0.55 mg PK/ml
PK-fresh conc	dilute PK-stock with equal volume of PBS → 5.5 mg PK/ml

Pefa-stock	Pefabloc SC (AEBSF) hydrochloride [4-(2-aminoethyl)-benzene sulfonylfluoride hydrochloride] (Roche - 11585916), 30 mg in 1 ml water. Immediately after dissolving aliquot 100 µl portions and store at -20 C (retains activity for 2 mo)
Pefa-fresh	Pefa-stock diluted 10x with water, prepared directly before application
1-propanol	HPLC quality, from Sigma Chromasolv 34871
LB	loading buffer for SDS-PAGE, containing Na- or Li- dodecylsulfate, glycerol, BPB, reducing agent, and buffer. All at a 2x concentration; to be used to mix with equal volume of test sample
LB1/1	mix LB with water 1/1; to be used for empty lanes or for dissolving precipitated PrP^{res} pellets.
TBS	8g NaCl, 0.2g KCl, 3g Tris, dissolve in ~900ml water, bring to pH7.4 with ~2ml HCl concentrated; final volume 1000ml with water
TBST	0.5ml Tween-20 per 1000ml TBS

Blocking buffer	3% skimmed milk powder in TBST (like Blotto, Santa Cruz, cat.# sc-2324).
1st Antibodies	Diluted in TBST. group B: L42, use at 0.1 µg/ml (RIDA R8005 from R-Biopharm, Darmstadt, Germany). group A: 12B2 at 0.2 µg/ml (from Central Veterinary Institute of Wageningen UR at Lelystad, The Netherlands). group C antibodies: SAF84 at 0.5 µg/ml (A03208 from SpiBio, France). 94B4 at 0.5 µg/ml (from Central Veterinary Institute of Wageningen UR at Lelystad, The Netherlands).
2nd antibody	Rabbit anti-mouse Ig conjugated to alkaline phosphatase (DAKO: D0314) Use at 1/5000 when developing with CDP* as chemoluminescent substrate.

Protocols A-D.

A: Protocol for preparing homogenates from brain tissue.

1. Weigh ~0.5 g brain tissue.
2. Add 5 ml of lysis buffer (or homogenisation buffer from Prionics®-Check Western BSE kit).
3. Homogenise with homogeniser of your choice (as an example of the procedure at Lelystad - the Consul FASTH instrument and disposable Prypcon homogenisation vials; 45 sec at 20000 rpm at RT). Let settle down for 15 min (if there are still visibly particles present a 2nd homogenisation could be carried). Before storage in freezer (!): transfer homogenate into a 15 ml Falcon tube and remove coarse debris by spinning at 500xg for 5min@RT. Collect supernatant and store at -20°C.

If desired, store also the pellet at -20°C e.g. for DNA preparation and genotyping.

B: Protocol for preparation of PrP^{res} and discrimination of bovine BSE-types by using PrP-site specific antibodies from group A and B.

This protocol consists of preparation of PrP^{res}, precipitation, and Western blotting using two PrP-site specific antibodies. The step removes cross-reactive components like PK and tissue matrix components. The procedure yields a near 100% recovery of PrP^{res}, and removes components that might alter migration. General: work in 500 µl vials; mixing is performed with microtip pipets, tilting or tipping the vials.

Preparation of PrP^{res}:

1. Digest 100 µl homogenate: add 10 µl of PK-fresh, mix, incubate for 60min@37°C. Stop: by adding 10 µl of Pefa-fresh.
2. Add an equal volume of 1-propanol to digest: thus e.g. 100-120 µl 1-propanol to 120 µl of digest. Vortex briefly.
3. Spin at 16000xg for 5min@RT.
4. Carefully remove (use pipet with 200 µl microtip) and discard the clear supernatant (~1µl pellet and ~4µl remaining supernatant left).

5. Add 100 µl LB1/1. Carefully redissolve the pellet by scratching with the pipet-tip and pipetting up-and-down several times. After there are no solid pellet parts visible in the solution anymore, still continue pipetting up-and-down for at least 10 times.
6. Incubate sample for 5-10min@96 C.
7. Take the sample out of the thermostat incubator. Let vials cool down. Mix.
8. Centrifuge 5min@16000xg@RT to pellet undissolved debris
9. Without mixing take a 10µl of supernatant for a slot of a 15-wells or 17-wells SDS-PAGE gel (compares with 500 µg TE).

Discrimination of bovine BSE-types by using PrP-site specific antibodies from group A and B in a parallel blot:

10. Have PrP^{res} of C-type BSE sample as reference.
11. Perform parallel SDS-PAGE for two blots with the same sample organisation and TE loading. As standards use a mixture of Invitrogen markers: 0.5 µl Magic Mark XP, and 5 µl SeeBlue plus 2 per lane (see example Fig 1A).
12. After electrotransfer: mark on both blots with a pencil the positions of the visible SeeBlue markers.
13. Stain one blot with 12B2 (0.2 µg/ml) and the other with L42 (0.1 µg/ml). Use for both blots exact the same conditions of incubation times, secondary antibody-alkaline phosphatase conjugate and film exposure times.
14. Discriminative features (see table "visual criteria" in Fig. 1):

a. L-type is characterized by: no binding by 12B2; and < 50% di-glycosylated fraction of PrP^{res} using L42.
b. H-type is characterized by: a nearly as high staining intensity of PrP^{res} with L42 and 12B2; and >50% di-glycosylated fraction of PrP^{res} using L42.
c. C-type is characterized by: a poor binding to 12B2 compared to L42; and >50% diglycosylated of PrP^{res} fraction using L42.
d. Migration position of non-glycosylated PrP^{res} is also indicative for H-type which is higher in the blot than that in C-type and L-type; for L-type and C-type the migration difference is less obvious and therefore difficult. Thus, differentiation above in a-c using the two antibodies is decisive for bovine BSE typing.

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TSE EURL CONTACT DETAILS

For any issue related to discriminatory tests please contact Romolo Nonno or Laura Pirisinu at:

romolo.nonno@iss.it

laura.pirisinu@iss.it

For other issues please contact the TSE EURL general mailbox:

EURL.TSE@izsto.it

This document is largely based on a previous one that originally was made available by APHA (UK) as EURL for TSEs. After the transition of the EURL to our consortium, in the documentation that we are making available, some minor changes were needed to update information, contacts and references.