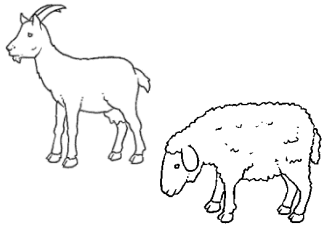
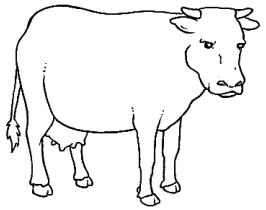
2021 round of TSE EURL EQAs: Results feed-back

19th TSE EURL/NRL Annual Meeting Turin - Italy 17 – 18 October 2022



DISCRIMINATORY WESTERN BLOT IN SMALL RUMINANTS EQA (DS21)

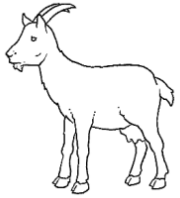


DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Laura Pirisinu
ISS - Rome

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

Istituto Superiore di Sanità - Rome



DISCRIMINATORY WESTERN BLOT IN SMALL RUMINANTS EQA (DS21)

Laura Pirisinu

ISS - Rome

EURL-TSE

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

Istituto Superiore di Sanità - Rome

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

Planning

- ❑ **19 Labs** expressed interest to partecipate in EQA round 2021 for Discriminatory WB in small ruminants
- ❑ Each NRL has been identified with a code (different for each EQA)
- ❑ Each round included 5 samples of frozen brainstem homogenates (50/50 mix of tissue and distilled water)
- ❑ The samples were stored at -80°C until shipment on dry ice
- ❑ All NRLs received an email (7th December 2021) informing the imminent dispatch of samples for the 6th December 2021 (!)
- ❑ Forms for returning the results were attached (and also supplied in the box in paper format)
  - Cover Letter (instructions for performing the proficiency test, information on safety, execution times and contacts)
  - Electronic form for submitting the results
- ❑ The deadline was 25/02/2022

The electronic form for submitting result must be uploaded with the link:
https://docs.google.com/forms/d/e/1FAIpQLSeV7b7MFKZWli-ZbtGflow7pjqFglgpEht6l43OXe7DbpxlGw/viewform?usp=sf_link

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

Samples dispatched

- ❑ 5 samples of frozen brainstem homogenates (50/50 mix of tissue and distilled water)
- ❑ Tested for homogeneity and stability

Sample ID	Expected results	Genotype	Sample origin/characteristics
DS2101	**Classical scrapie**	ARH/VRQ	Pool of classical scrapie samples from sheep (UK Reference Material)
DS2102	**Classical scrapie**	VRQ/VRQ	Classical scrapie sample from sheep (UK RM)
DS2103	**Classical scrapie**	ARH/VRQ	Pool of classical scrapie samples from sheep (UK RM)
DS2104	**BSE not excluded**	ARQ/ARQ	Pool of samples from sheep with experimental BSE (UK and IT RM)
DS2105	**Classical scrapie**	ARQ/VRQ	Pool of classical scrapie samples from sheep (UK RM)

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

Results tabulation (sent 06/07/2022)

N° of methods used per Lab	Total
one test method	14
two test methods	4
Total partecipating Laboratories	**18**

Test method used	N° of Labs
APHA Bio-Rad TeSeE-based Hybrid Western blotting Method	13
APHA Prionics-based Hybrid Western blot Method	2
Bio-Rad Discriminatory Test (based on the CEA Discriminatory Western blot Method)	3
FLI Discriminatory Western blot Method	1
ANSES Discriminatory Western blot Method	1
ISS Discriminatory Western blot Method	1

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

Results

- **<u>All Laboratories reported the correct results</u>**

But, EURL contacted 3 Laboratories:

- A Lab didn't send the results in the due time (**Lab 143**)
- The excel file compiled with some unexpected modification of the name of the method used (**Lab 352**)
- The declared method did not correspond to the reagents and raw data (**Lab 910**)
- The **Lab 352** used, as 1st alternative test, a method that did not correspond to any protocol reported on the "TSE Strain Characterisation in small ruminants – A Technical handbook for NRL in the EU"

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

Actions undertaken

- ❑ The Lab 143 informed the EURL not to participate to this PT round
- ❑ Labs 352 and 910 were asked to provide clear information on the method/s used.

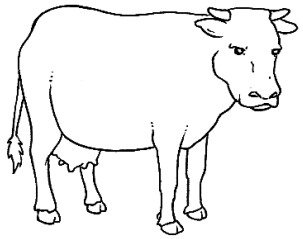
  → The info provided were satisfactory
- ❑ The alternative method used by Lab 352 should not be used for discriminatory testing

DISCRIMINATORY WB IN SMALL RUMINANTS EQA (DS21)

All Laboratories (18) passed the PT!

1. Belgium	10. Netherlands
2. Bulgaria	11. Poland
3. Czechia	12. Portugal
4. France	13. Romania
5. Germany	14. Slovakia
6. Greece	15. Slovenia
7. Hungary	16. Spain
8. Ireland	17. Switzerland
9. Italia	18. United Kindom

WELL DONE!



DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Laura Pirisinu
ISS - Rome

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

Istituto Superiore di Sanità - Rome

DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Planning

- ❑ **11 Labs** expressed interest to partecipate in EAQ round 2021 for Discriminatory WB in bovine
- ❑ Each NRL has been identified with a code (different for each EQA)
- ❑ Each round included 4 samples of frozen brain homogenates (50/50 mix of tissue and distilled water)
- ❑ The samples were stored at -80°C until shipment on dry ice
- ❑ All NRLs received an email (7th December 2021) informing the imminent dispatch of samples for the 6th December 2021 (!)
- ❑ Forms for returning the results were attached (and also supplied in the box in paper format)
  - Cover Letter (instructions for performing the proficiency test, information on safety, execution times and contacts)
  - Electronic form for submitting the results
- ❑ The deadline was 25/02/2022
- ❑ Note: the samples of the EQA for Discriminatory Western Blot In Small Ruminants in the same box

DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Samples dispatched

- ❑ 4 samples of frozen brainstem homogenates (50/50 mix of tissue and distilled water)
- ❑ Tested for homogeneity and stability

Sample ID	Expected results	Sample origin/characteristics
DB2131	C-BSE	UK Reference Material
DB2132	L-BSE	Pool of field isolates from IT
DB2133	H-BSE	Field isolate identified by Irish NRL (kindly provided by Ann Sharpe)
DB2134	C-BSE	UK Reference Material

DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Results (sent 22/07/2022)

Lab ID	Results
127	X
277	✓
472	✓
514	X
532	X
539	✓
567	✓
572	X
688	✓
803	✓
959	✓

7 of 11 NRLs returned the corrected results

Non-compliant results for 4 Labs

DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

Analysis of non-compliant results

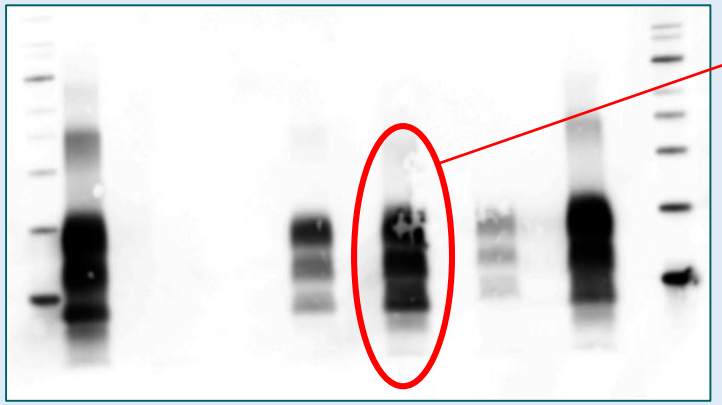
Sample ID	Expected results	Lab 127	Lab 514	Lab 532	Lab 572
DB2131	C-BSE	C-BSE	C-BSE	C-BSE	C-BSE
DB2132	L-BSE	C-BSE	L-BSE	L-BSE	L-BSE
DB2133	H-BSE	H-BSE	L-BSE	negative	negative
DB2134	C-BSE	C-BSE	C-BSE	C-BSE	C-BSE

Actions:
Analysis of raw data by EURL
The Labs were asked to re-test the samples, suggesting to revise the protocol used
with the support of the EURL guide “Approach for the provisional classification of bovine TSE isolates”.

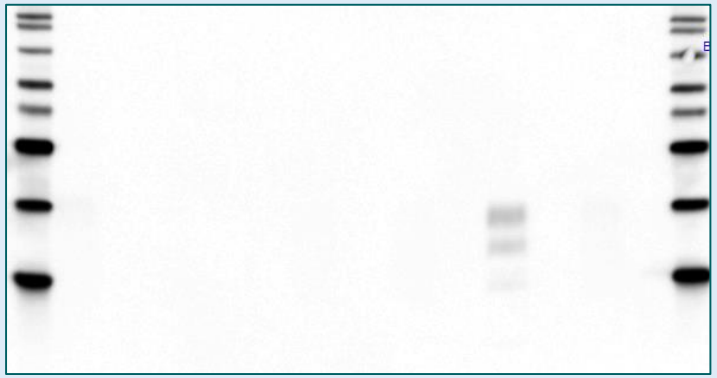
Non compliant results for Lab 127

APHA Biorad Hybrid WB (sha31, P4)

1st test



sha31

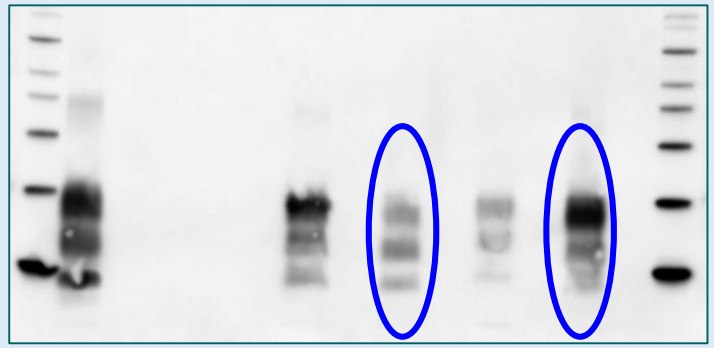


P4

DB2132: C-BSE
Instead of L-BSE

The Lab repeated the test following 1:10 dilution of the strong samples
→ All correct results!

2nd test



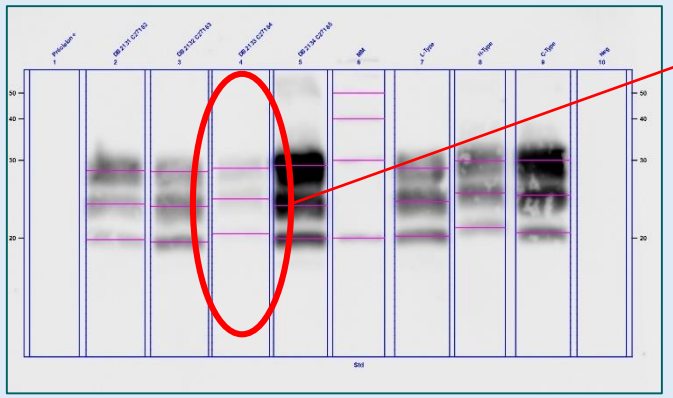
sha31

Sample ID	Expected results	Lab 127
DB2131	C-BSE	C-BSE
DB2132	L-BSE	L-BSE
DB2133	H-BSE	H-BSE
DB2134	C-BSE	C-BSE

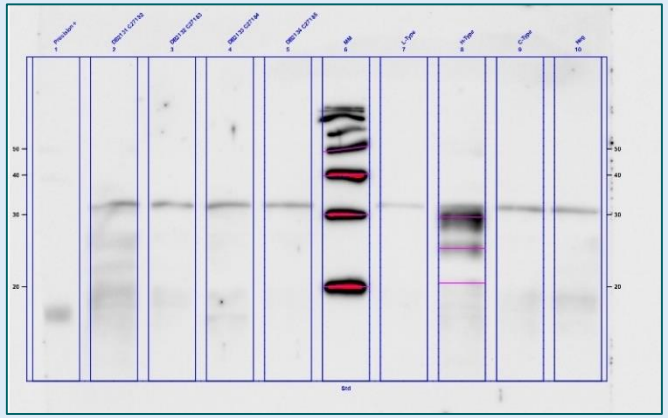
Non compliant results for Lab 514

APHA Biorad Hybrid WB (sha31, P4)

1st test



Sha31

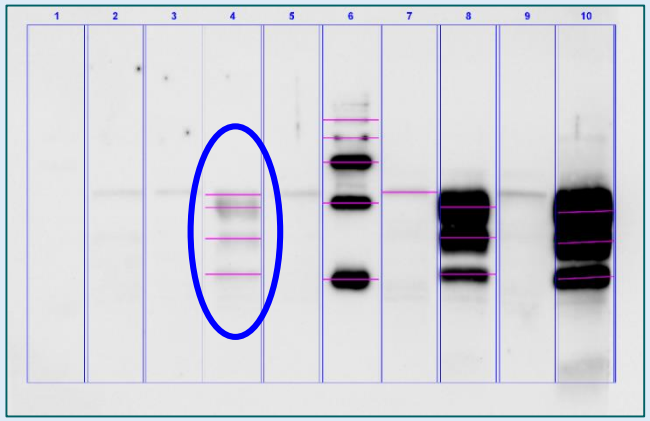


P4

DB2133: L-BSE
instead of H-BSE

The Lab repeated the test changing P4 with 12B2
→ All correct results!

2nd test



12B2

Sample ID	Expected results	Lab 514
DB2131	C-BSE	C-BSE
DB2132	L-BSE	L-BSE
DB2133	H-BSE	H-BSE
DB2134	C-BSE	C-BSE

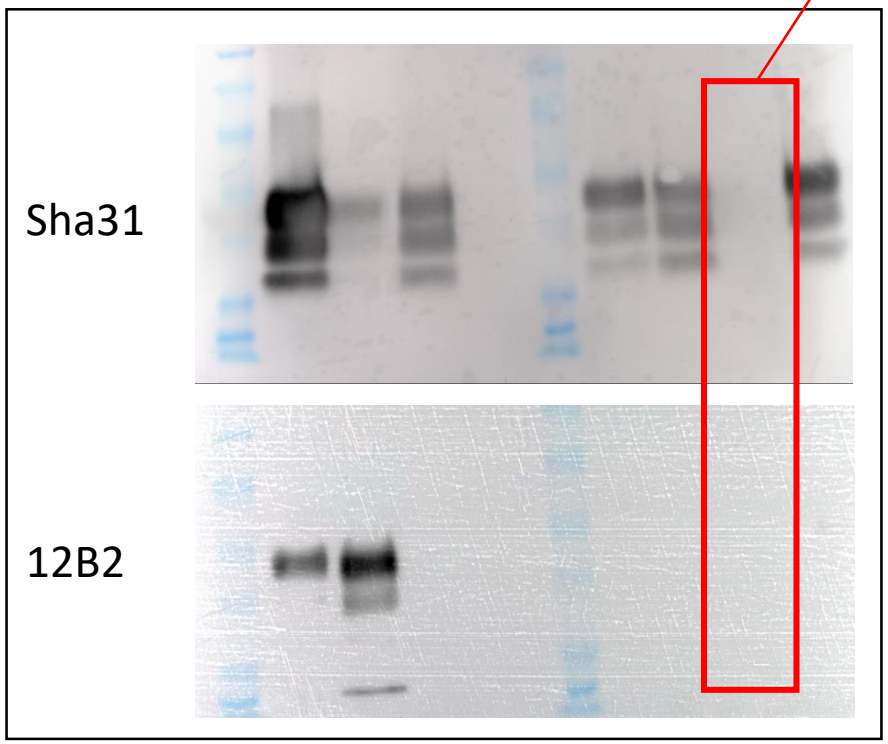
Non compliant results for Lab 532

In house WB BioRad based 5x PK (sha31, 12B2)

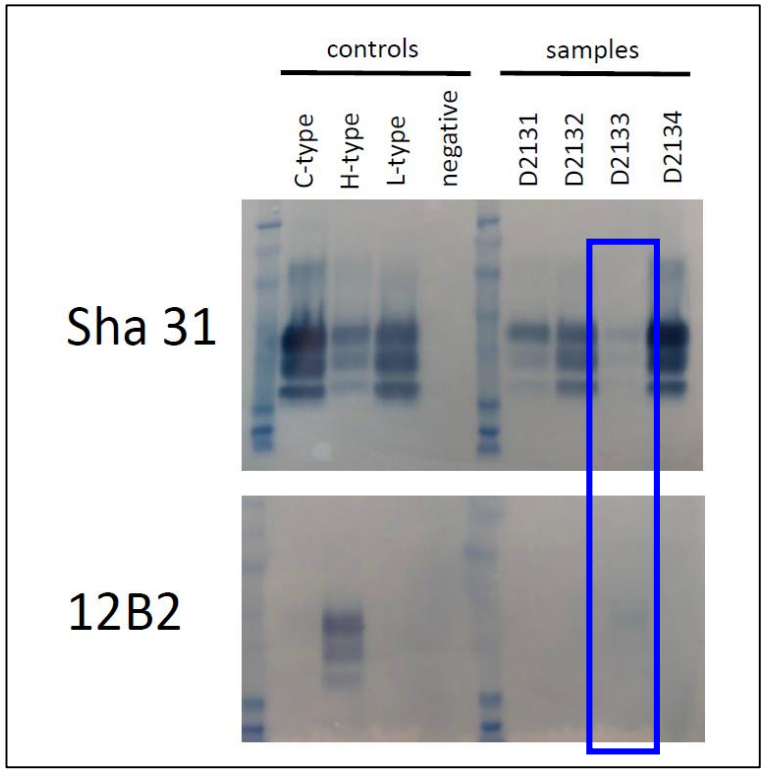
DB2133:
negative
Instead of H-BSE

The Lab repeated the test changing reagents
→ All correct results!

1st test



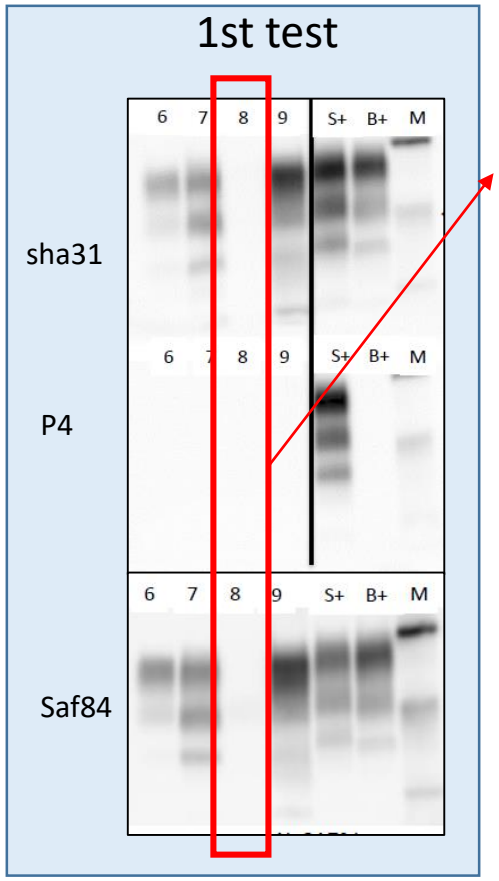
2nd test



Sample ID	Expected results	Lab 532
DB2131	C-BSE	C-BSE
DB2132	L-BSE	L-BSE
DB2133	H-BSE	H-BSE
DB2134	C-BSE	C-BSE

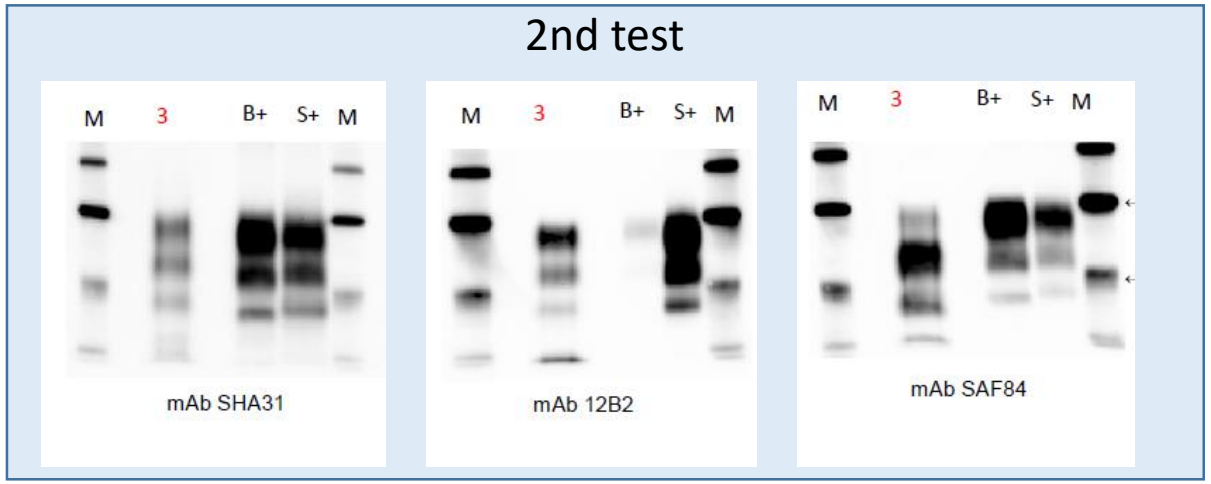
Non compliant results for Lab 572

APHA Biorad Hybrid WB (sha31, P4)



DB2133:
negative
instead of H-BSE

The Lab repeated the test, loading double of concentration compared to the original protocol and substituting P4 for 12B2 as the N-terminal antibody
→ All correct results!



Sample ID	Expected results	Lab 572
DB2131	C-BSE	C-BSE
DB2132	L-BSE	L-BSE
DB2133	H-BSE	H-BSE
DB2134	C-BSE	C-BSE

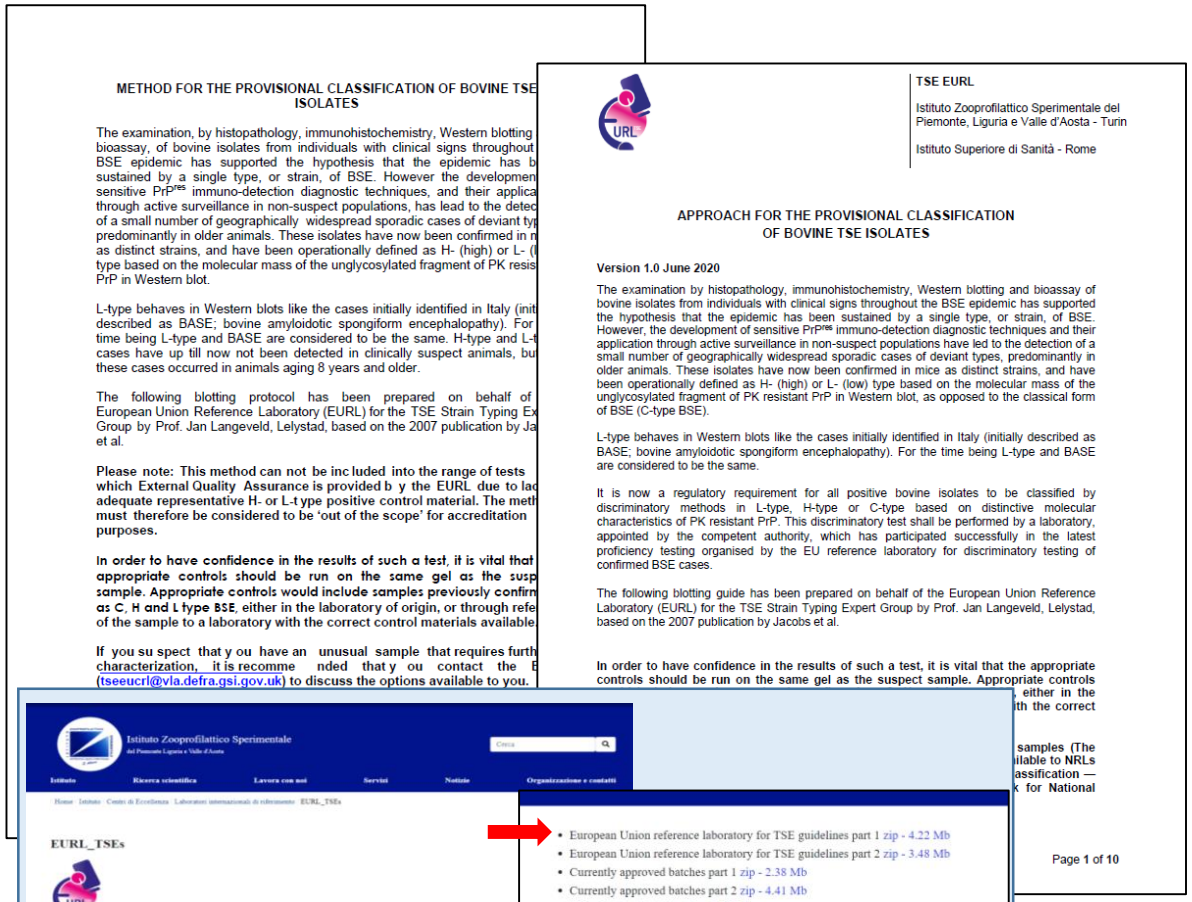
DISCRIMINATORY WESTERN BLOT IN BOVINE EQA (DB21)

All Laboratories passed the PT!

11 Laboratories

1. Belgium
2. France
3. Germany
4. Ireland
5. Italia
6. Netherlands
7. Poland
8. Portugal
9. Spain
10. Switzerland
11. United Kindom

Approach for the provisional classification of bovine TSE isolates



METHOD FOR THE PROVISIONAL CLASSIFICATION OF BOVINE TSE ISOLATES

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

L-type behaves in Western blots like the cases initially identified in Italy (init described as BASE; bovine amyloidotic spongiform encephalopathy). For time being L-type and BASE are considered to be the same. H-type and L-t cases have up till now not been detected in clinically suspect animals, bu these cases occurred in animals aging 8 years and older.

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

Please note: This method can not be inc luded into the range of tests which External Quality Assurance is provided b y the EURL due to lac adequate representative H- or L-t ype positive control material. The meth must therefore be considered to be 'out of the scope' for accreditation purposes.

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

If you su spect that y ou have an unusual sample that requires furth characterization, it is recomme nded that y ou contact the E (tseeucrl@vla.defra.gsi.gov.uk) to discuss the options available to you.

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

Version 1.0 June 2020

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 ..., either in the ...th the correct

...samples (The ...ilable to NRLs ...assification — ...k for National

Page 1 of 10

Istituto Zooprofilattico Sperimentale

Istituto | Ricerca scientifica | Lavora con noi | Servizi | Notizie | Organizzazione e contatti

EURL_TSEs

- European Union reference laboratory for TSE guidelines part 1 zip - 4.22 Mb
- European Union reference laboratory for TSE guidelines part 2 zip - 3.48 Mb
- Currently approved batches part 1 zip - 2.38 Mb
- Currently approved batches part 2 zip - 4.41 Mb
- EU widw batch testing zip - 3.96 Mb
- Test approved zip - 1.04 Mb
- Test protocols part 1 zip - 4.18 Mb
- Test protocols part 2 zip - 2.07 Mb
- Test protocols part 3 zip - 2.37 Mb
- Test protocols part 4 zip - 2.77 Mb

Soon on the new web site

https://admin.izsplv.it/it/istituto/212-centri-eccellenza/laboratori-internazionali-riferimento/422-eurl_tses.html

JOURNAL OF CLINICAL MICROBIOLOGY, June 2007, p. 1821–1829 Vol. 45, No. 6
0095-1137/07/$08.00+0 doi:10.1128/JCM.00160-07

Molecular Discrimination of Atypical Bovine Spongiform Encephalopathy Strains from a Geographical Region Spanning a Wide Area in Europe†▽

Jorg G. Jacobs,[1] Jan P. M. Langeveld,[1]* Anne-Gaëlle Biacabe,[2] Pier-Luigi Acutis,[3] Miroslaw P. Polak,[4] Dolores Gavier-Widen,[5] Anne Buschmann,[6] Maria Caramelli,[3] Cristina Casalone,[3] Maria Mazza,[3] Martin Groschup,[6] Jo H. F. Erkens,[1] Aart Davidse,[1] Fred G. van Zijderveld,[1] and Thierry Baron[2]

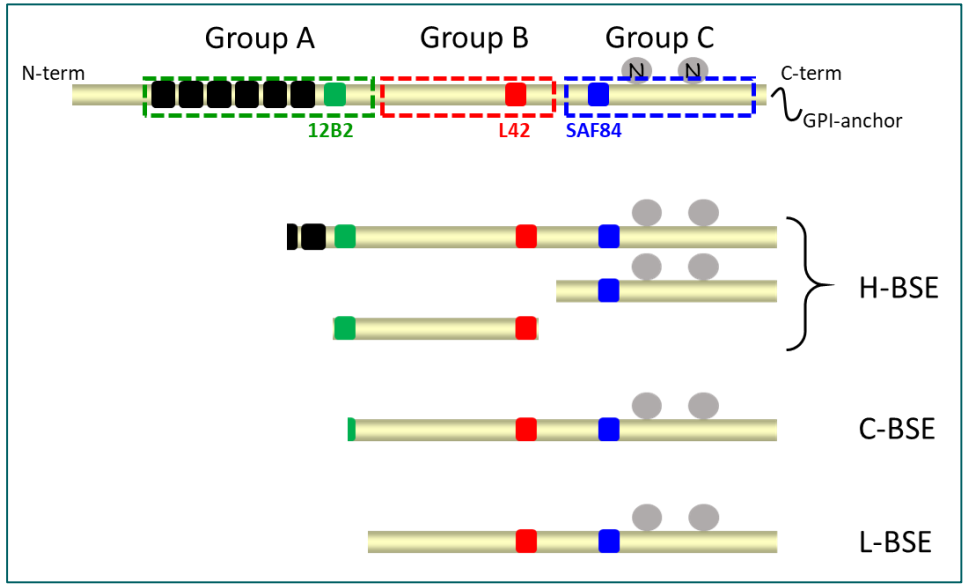
Central Institute for Animal Disease Control (CIDC-Lelystad), 8203 AA 2004, Lelystad, The Netherlands[1]; Agence Française de Sécurité Sanitaire des Aliments (AFSSA-Lyon Fr), Unité ATNC, 31 avenue Tony Garnier, 69342 Lyon cedex 07, France[2]; Centro di Referenza per le Encefalopatie Animali (CEA), Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Turin, Italy[3]; National Veterinary Research Institute (NVRI), Al. Partyzantow 57, 24-100 Pulawy, Poland[4]; National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden[5]; and Friedrich-Loeffler-Institut, Boddenblick 5a, 17493 Greifswald-Insel Riems, Germany[6]

The blotting protocol has been prepared on behalf of the EURL (UK) for the TSE Strain Typing Expert Group by Jan Langeveld, on the 2007 publication by Jacobs et al.

Method for the provisional classification of bovine TSE isolates

Classification of C-type, H-type and L-type BSE using the following parameters:

1. molecular migration of PrP^{res} bands
2. differential binding to PrP-specific antibodies
3. PrP^{res} glycoprofiles
4. number of non-glycosylated PrP^{res} bands
5. susceptibility to proteinase K (PK)



	1	2	3	4	5
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

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

Table II: grouping of antibodies for recognition of bovine BSE-types 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

Preferred antibodies because of high affinity

Method for the provisional classification of bovine TSE isolates

A 2-blot protocol for PrPres typing in BSE from cattle.

For standard discrimination of the 3 BSE types only 2 parameters are required, and can be performed by visual inspection using antibodies of **sufficiently high affinity** to detect PrPres:

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

2. Glycoprofile differences between L-type on the one hand, and C- and H-type on the other hand using L42 (group B antibody)

visual criteria

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

This double blot test yields sufficient criteria for discrimination of three types

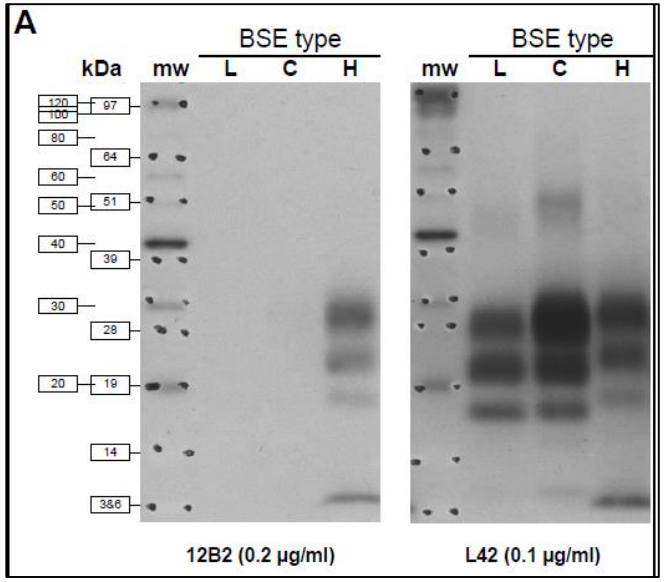
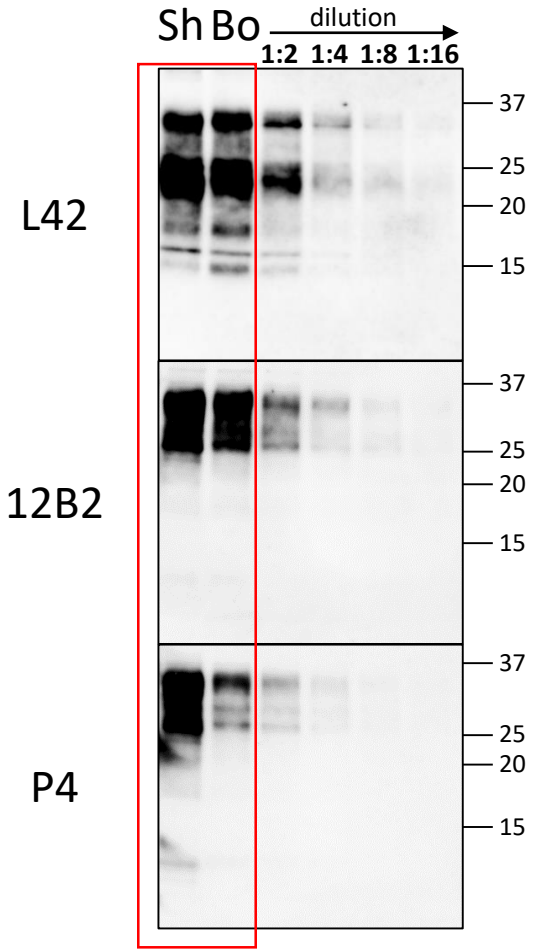


Fig by Method for the provisional classification of bovine TSE isolates

Affinity of N-terminal antibodies: P4 vs 12B2

PrP^{C} from healthy sheep and cattle



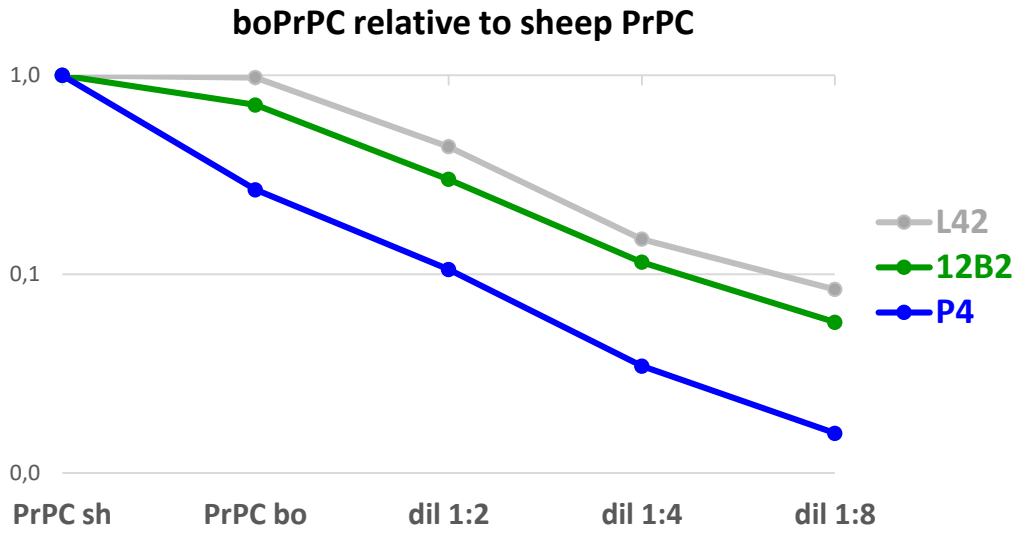
12B2 93-WGQGG-97

P4 93-WGQGGSH-99

Ovine PrP aa sequence 87-PHGGGGWGQGGSHSQWNK-104

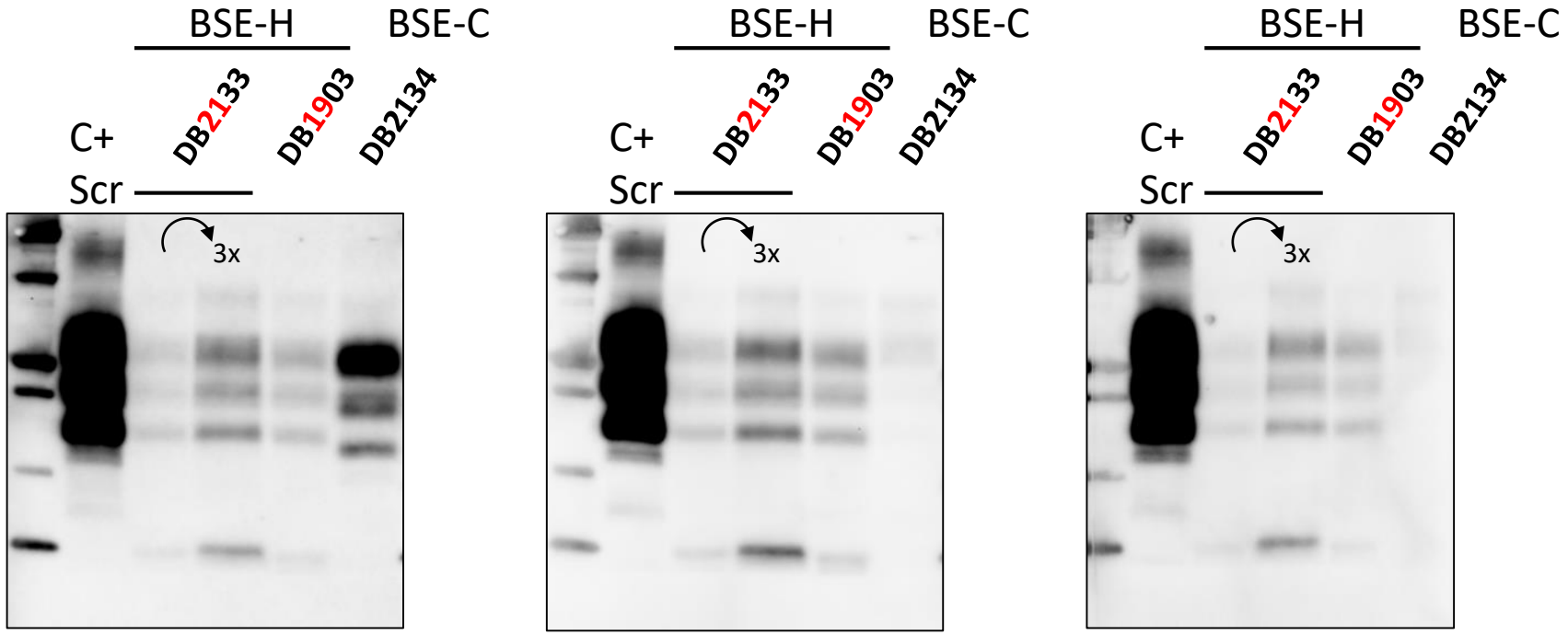
Bovine PrP aa sequence ----PHGGGGWGQGGTHSQWNK-----

Treonin vs Serine at 98 aa

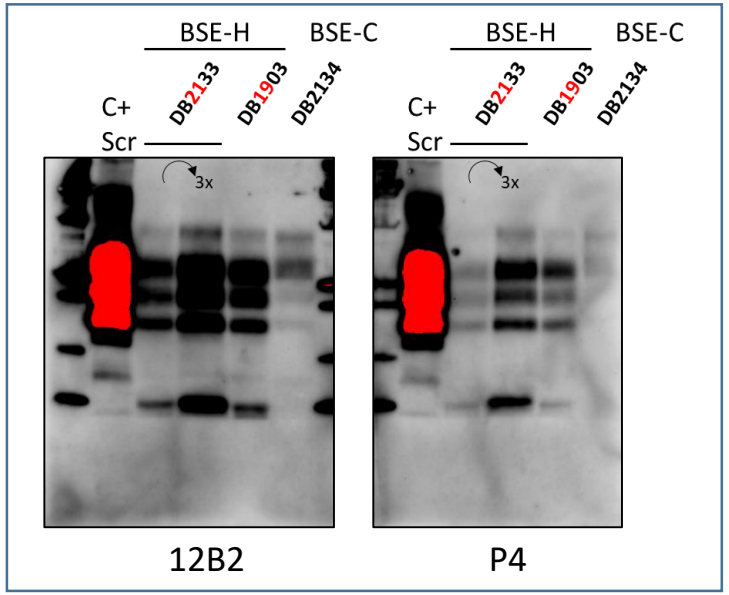


By Elena Esposito (ISS, Rome)

Affinity of N-terminal antibodies: P4 vs 12B2



Long exposure time

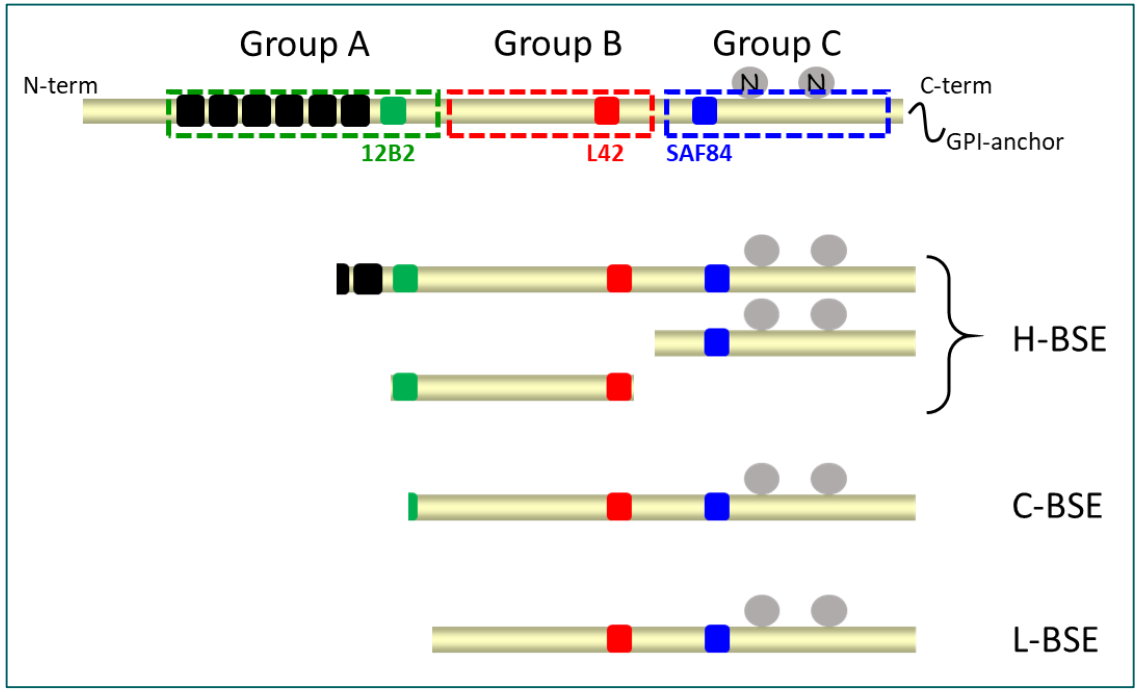


By Elena Esposito (ISS, Rome)

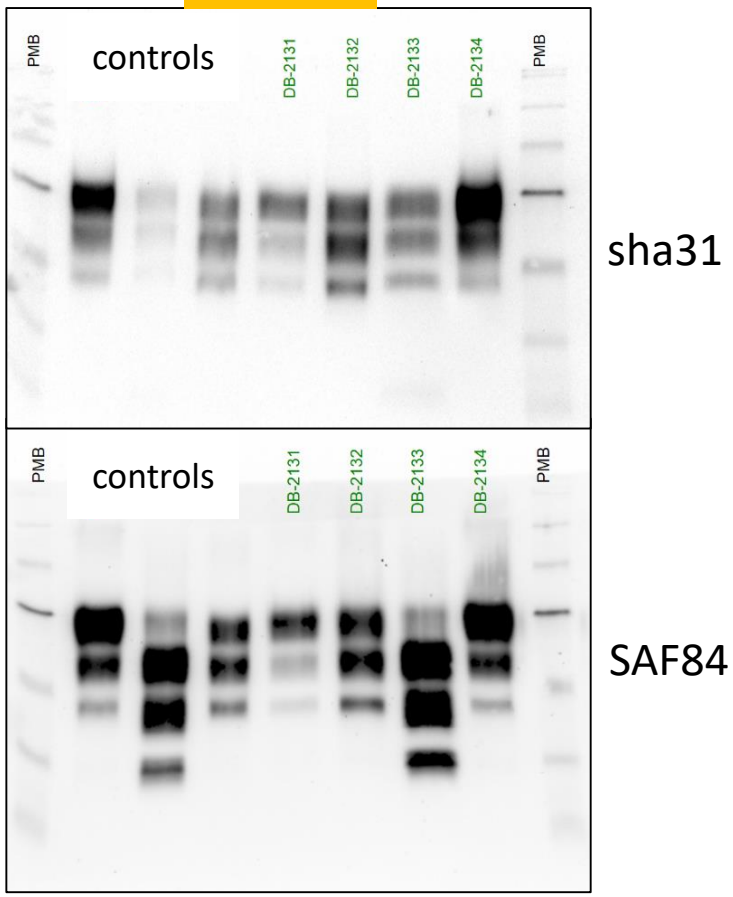
Method for the provisional classification of bovine TSE isolates

Other differences can be detected:

3. 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



Lab 277 (modified)



Method for the provisional classification of bovine TSE isolates

Other differences can be detected:

4. The susceptibility of C-type for proteinase K is nearly the same between mild or stringent digestion condition, while on the contrary PrPres of L-type and H-type hardly survives the stringent condition.

Lab 567 (modified)

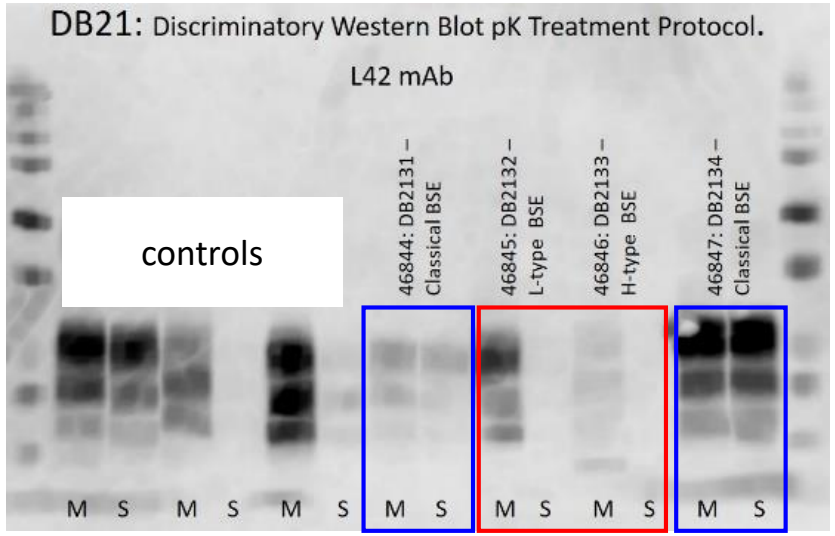


Fig by Jacobs et al 2007

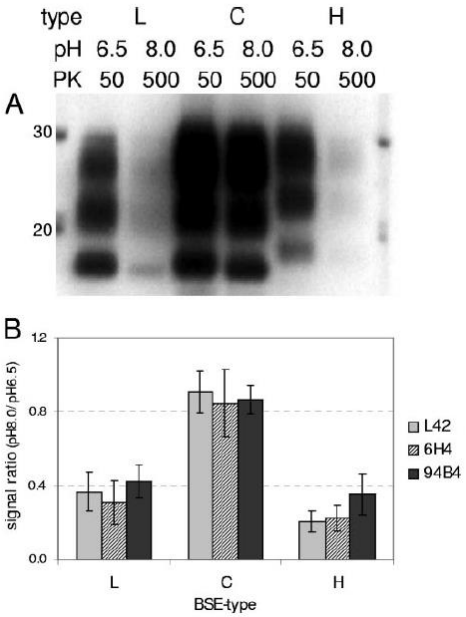
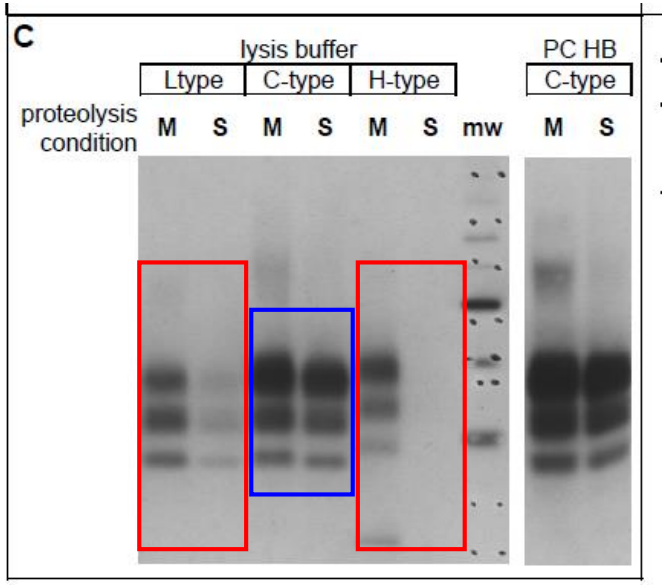


Fig by Method for the provisional classification of bovine TSE isolates



DISCRIMINATORY WESTERN BLOT IN BOVINE – Troubleshooting

- Two samples were not correctly identified:
  - DB2132 was a sample of L-BSE with strong signal
  - DB2133 was a sample of H-BSE with weak signal

- The selection of the samples for PT rounds is based on the best quality of available samples in terms of characteristics and signal, however the shortage of BSE types in EU limited/limits the choice

- The two samples were field isolates detected and confirmed by 2 NRLs (IT and IRL)
  → Represent the natural variability that may occur during the surveillance activity

DISCRIMINATORY WESTERN BLOT IN BOVINE – Troubleshooting

Some tips:

- ✓ **Strong signals** → strong positive samples may give a saturated signal intensity which may obscure visual interpretation of the banding profile, in particular of glicoprofile interpretation. (dilute the samples and/or use short time exposure image)
  - Possible mistake: L-BSE vs C-BSE

- ✓ **Use of P4** → consider the low affinity for boPrP (if you have weak samples or low sensitivity of your WB)
  - Possible mistake: H-BSE as C-BSE/L-BSE

- ✓ **Use of strong PK conditions** → consider that H-BSE and L-BSE are more susceptible to strong PK digestion than C-BSE (if you have weak samples or low sensitivity of your WB)
  - Possible mistake: H-BSE and L-BSE as negative

- ✓ **Use of 12B2** → consider that you could see a small amount of C-BSE (it depends on the sensitivity of your WB and digestion conditions)
  - Possible mistake: C-BSE as H-BSE (in particular if you are familiar with P4 where the lanes are usually clear due to low sensitivity!)

Last suggestion: always compare the signals obtained with core-mAb and N-terminal one. The use of an internal control that is detected in the same way (quantitity) by both mAbs may be helpful (for example classical scrapie)!

DISCRIMINATORY WESTERN BLOT METHODS

SMALL RUMINANTS

Discriminatory testing must be performed following the protocols and the procedures as reported in the Technical handbook:

"TSE Strain Characterisation in small ruminants – A Technical handbook for NRL in the EU"

→ No changes are allowed

BOVINE

- There is no a defined protocol

- EURL guide: «Approach for the provisional classification of bovine TSE isolates"
  → Parameters and characteristics useful to set up a discriminatory WB

- 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 Technical handbook for SR
  → Use of Sha31 and P4: it is possible to make changes (P4→12B2)

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- Elena Esposito for WB

- All partecipants to PTs

- Labs with non-compliant results for being responsive, collaborative and open to confrontation