

Spongiform Encephalopathy Advisory Committee

Date: 11 September 2002

Title of Paper

Situation Report on VLA Programme of Molecular Typing of Scrapie Isolates Using the Prionic Western Blot

Issue

The VLA provided SEAC (November 2001 meeting) with a pre-publication report on the modified format of the Prionics Check Western Blot to distinguish between scrapie and experimental BSE in sheep. This work has now been published. The VLA was asked to submit a plan for the evaluation of the performance of the test, and for retrospectively testing of material collected in recent years. This paper provides an update on the work in progress with respect to the test evaluation. Specifically, it details statistical analyses which have been performed on the test results with respect to repeatability, and the identification of statistical significant differences to identify different strains.

Action Required from Committee

- Members are asked to state whether they agree with the conclusions (see point 3) of Phase 1 (the evaluation of the repeatability of the test to confirm its suitability for strain discrimination)
- Members may wish to comment on the SSC strategy and its impact on the VLA's test evaluation process

List of material attached

- [Summary paper](#) on progress to date – including conclusions on Phase 1 (the evaluation of the repeatability of the test)
- [Annex 1](#) – Published paper by Stack *et al.* (2002)
- [Annex 2](#) – Statistical analysis of molecular weights and glycoform ratios of the data supplied for the Acta Neuropathologica scientific paper.
- [Annex 3](#) – Validation of the hybrid western immunoblotting technique for TSEs in sheep – summary document
- [Annex 4](#) (external website) – Strategy to investigate the possible presence of BSE in sheep – adopted by the Scientific Steering Committee at its meeting of 4-5 April 2002.

SITUATION REPORT ON VLA PROGRAMME OF MOLECULAR TYPING OF SCRAPIE ISOLATES USING THE PRIONICS WESTERN BLOT

1. Introduction

At meeting 71 in November 2001, SEAC received a pre-publication briefing from M Stack of the Veterinary Laboratories Agency on the use of a modified format of the Prionics Check Western blot to distinguish between scrapie and experimental BSE in sheep (paper 71/5).

As a result of that meeting, the VLA was asked to submit a plan for the evaluation of the performance of the test, and for retrospectively testing of material collected in recent years. This plan was submitted to DEFRA and subsequently discussed, and agreed, at SEAC meeting 73 on 10 April 2002 (paper 73/4). The first phase of the plan involved the evaluation of repeatability of the test to confirm its suitability for strain discrimination.

This paper reviews available data and provides a situation report on progress.

2. Published data

The data presented in November has now been published. The full paper is appended (annex 1). That the data required validation was recognised at the time, especially given the limited amount of ovine control material infected with BSE that was available as positive control. Nevertheless, it is unfortunate that the presentation to SEAC was not accompanied by a statistical analysis. That analysis is appended to this document (annex 2).

In summary the analysis suggests that, on the basis of the limited amount of data presented:-

- The molecular weight of the unglycosylated band of BSE and CH1641 appear to be sufficiently different (lower) than scrapie to enable discrimination. The smallest difference between groups was between BSE in sheep and ARQ/ARQ Cheviot scrapie at 0.6kd, while CH1641 and ARQ/ARQ Cheviot scrapie was 1.0kd
- The ratio of signals of di- to mono-glycosylated bands appears to allow discrimination between ovine BSE and CH1641.

The differences were statistically significant.

3. Phase 1 - Repeatability data

A study to measure the inherent variability within the testing methodology was planned using homogenised brain stem from a scrapie affected sheep of ARQ/ARQ genotype. BSE in sheep was not included in this study as it is still too scarce a resource, and would be needed for later stages of these studies. The basic study used:-

- The use of a single brain pool, sub-aliquoted and frozen for the primary evaluation
- 2 operators to study between operator variability
- the operators read their own and each other's gels blind.
- A third party (Anne-Gaelle Biacabe, AFSSA, Lyon) read all gels blind, and also re-coded all gels so that they could be re-read by operators 1 and 2.
- Each operator set up a total of 10 gels, half on day 1, half on day 2 to study between gel and between day variation

- a small study of the effect of autolysis was also conducted, using brains from six sheep with the following genotypes (ARQ/ARQ; VRQ/VRQ (two); ARQ/VRQ; ARQ/AHQ; ARH/VRQ). The brains were homogenised either on day 0 or on day 4, the latter after incubation at 25°C for four days.
- The full report has been submitted to DEFRA, but the executive summary is appended at annex 3.

Repeatability conclusions

In summary the study showed that:-

- The coefficient of variation for molecular weight measurement was less than three percent. Apart from residual error, the differences between gels and between days made the largest contribution to variation. The differences between operators and readers were smaller, and with one exception were responsible for less than one per cent of total variance
- Measurement of band density for the different glycoforms was more problematic, with coefficients of variation exceeding 10%. This was most notable for the unglycosylated band.
- The coefficient of variation for the ratio of di- to mono-glycosylated bands was 26%. Measurement of this ratio was considered to be less useful than molecular weight measurement for strain discrimination, other than in distinguishing ovine BSE from CH1641.
- Between-gel variation should be compensated for by the inclusion of standard controls on all gels, ideally BSE in sheep, but also bovine BSE and ovine scrapie.
- Using fresh brain samples, the genotype of the source sheep did not result in statistically significant variations in molecular weights of bands.
- Molecular weights of samples tested fresh were usually higher than for samples that were autolysed in the laboratory. The differences, although small, were statistically significant for mono- and un-glycosylated bands.
- This study did not investigate the effect of autolysis on staining patterns using monoclonal antibody P4, which does otherwise appear to discriminate between BSE and scrapie in sheep. It will be necessary to show that autolysis does not produce a “BSE-like” signature with scrapie.
- It will also be necessary to take into account the likely behaviour of BSE in genotypes of sheep that have not been examined so far (and are not yet available experimentally)

Conclusion

- a) the method is appropriate for use in the retrospective study – with care – to discriminate between BSE in sheep (as currently recognised) and scrapie
- b) any preliminary suggestion of a BSE-like signature, on a gel carrying bovine BSE as a control, should lead to further testing using ovine-BSE and scrapie on the same gel

4. Phase 2 – retrospective testing

The plan for the use of the method for retrospective testing of samples has been discussed by SEAC at its meeting of 10th April 2002 (paper 73/4).

A preliminary requirement for such an evaluation, in the light of the above analysis and in order to assist interpretation of results, is the gathering of raw data for all samples and the quantification of samples remaining for evaluation. Some samples

have been used in strain typing projects at the VLA, so this has required the recall of materials no longer required in such projects, and collation of data. This has taken longer than expected, but the database created is now sufficiently well populated to enable a structured testing programme to be planned.

As has previously been agreed with SEAC, the testing will proceed to completion before results are released. This will enable a full interpretation of the outcome, given the considerable uncertainty that still surrounds the likely behaviour of BSE in different genotypes of sheep, and of autolysis on the signature of scrapie and BSE in sheep.

That agreement is underpinned by a recognition that:-

- a) genotypes that are most likely to carry a strain that can be recognised as BSE (ARQ/ARQ) will be tested first, and
- b) that any isolate in which the preliminary indicators suggest that the isolate may be BSE (including behaviour in mouse bioassay), will result in prioritisation of re-testing and the application of additional molecular methods (including immunocytochemistry)
- c) that any isolate in which all the indicators represent **a confident interpretation** that the isolate is BSE (including behaviour in mouse bioassay), will warrant the reporting of results before the study is complete (**see also below**)

5. SSC Strategy to investigate the possible presence of BSE in sheep

In April 2002 the Scientific Steering Group adopted a strategy, which correctly highlighted the many uncertainties that surround methods of strain discrimination by molecular means. The VLA, in its capacity as Community Reference Laboratory, has subsequently received a request from the European Commission to translate the document into instructions for all National Reference Laboratories.

This is not going to be easy, especially given the early status of much of the research. It also requires close collaboration between several research groups. Although this was already planned for prospective work, this will also have to be considered in the context of the retrospective testing that has now started.

The Strategy, and the European Commission, envisage:-

- a) the establishment of ring trials on any material that has a “BSE-like” signature by molecular methods
- b) the establishment of a review panel, comprising the collaborators in the comparison molecular methods, to interpret results

Plans for this evaluation have not been finalised, but the approach is clearly likely to impose on the DEFRA/SEAC evaluation process. It does however have the added benefit of speeding up the evaluation processes, and may conclude that any significant finding (such as the identification of a “BSE-like” signature) does result in full agreement between methods.

The development of instructions for other National Reference Laboratories cannot proceed until 2003, but the establishment of preliminary procedures for ring trials and evaluation of results will proceed before this.

D Matthews.

Annex 1

REFERENCE:

Chaplin M J, Clark J and Stack M J

Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH 1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies.

Acta Neuropathol (2002) 104: 279 - 86

Statistical analysis of molecular weights and glycoform ratios of the data supplied for the Acta Neuropathologica scientific paper.

Comparisons of sample means

Analyses of variance were done on the data in each of the six tables using a model with main effects for sample and replicate. In all cases the overall differences between the sample means were highly significant ($p < 0.001$). All pairs of individual sample means within a table were then compared using the Bonferroni test and the results are presented in tables 1-6 below as homogeneous groupings. The sample means are presented in order of magnitude and each subsequent column indicates by '****' a group of adjacent samples which are not significantly different by this test at $p = 0.05$.

The Bonferroni test is often used to avoid obtaining too many 'significant' differences just by chance when the differences between all pairs of means are tested. It is based on the usual t-test but the number of comparisons made divides the probability level for significance for individual comparisons. For example with 12 means as below there are $12 \times 11 / 2 = 66$ pairs of means and therefore a single difference would only be considered significant if its p-value was less than $0.05 / 66 = 0.00076$. In this way the overall significance level for the set of comparisons is maintained at $p = 0.05$.

Table 1: Molecular Weights(kD) of Di-glycosylated Band

Bonferroni test Homogenous Groups, alpha = .050 Error MS = .07257, df = 77							
Cell No.	Sample	Mean	1	2	3	4	5
3	CH1641	25.15438	****				
4	ChevBSE1	25.73800		****			
5	ChevBSE2	26.03187		****	****		
11	SSBP/1	26.10663		****	****	****	
6	RomBSE1	26.19200		****	****	****	****
2	ChevARQ	26.20175		****	****	****	****
7	RomBSE2	26.31550			****	****	****
1	RomVRQ	26.38975			****	****	****
10	Swale	26.43562			****	****	****
12	BSE2	26.47363			****	****	****
8	BSE1	26.56688				****	****
9	ChevVRQ	26.63775					****

'CH1641' is in group 1 on its own and therefore differs significantly from all the samples below it in the table. 'ChevBSE1' down to 'ChevARQ' form group 2 and these samples do not differ significantly from each other. This group and the remaining groups overlap, so that for example, 'SSBP/1' doesn't differ from any of the samples between 'ChevBSE1' and 'BSE1'.

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Table 2: Molecular Weights(kD)of Mono-glycosylated Band

Bonferroni test Homogenous Groups, alpha = .050 Error MS = .07972, df = 77								
Cell No.	Sample	Mean	1	2	3	4	5	6
3	CH1641	21.37763	****					
4	ChevBSE1	21.67312	****	****				
6	RomBSE1	21.86738	****	****	****			
5	ChevBSE2	21.96188		****	****	****		
7	RomBSE2	22.02137		****	****	****		
12	BSE2	22.28750			****	****	****	
8	BSE1	22.38388				****	****	
2	ChevARQ	22.44938				****	****	
1	RomVRQ	22.52663					****	
11	SSBP/1	22.66012					****	****
10	Swale	22.71038					****	****
9	ChevVRQ	23.07350						****

In this table the highest valued BSE-in-sheep sample, 'RomBSE2', is in group 4 with the lowest scrapie sample, 'ChevARQ', and hence they do not differ significantly, but there is no other overlap between groups containing these two types of sample.

Table 3: Molecular Weights (kD)of Un-glycosylated Band

Bonferroni test Homogenous Groups, alpha = .050 Error MS = .06188, df = 77									
Cell No.	Sample	Mean	1	2	3	4	5	6	7
3	CH1641	18.05375	****						
4	ChevBSE1	18.30800	****	****					
5	ChevBSE2	18.35850	****	****					
6	RomBSE1	18.36150	****	****	****				
7	RomBSE2	18.40450	****	****	****				
12	BSE2	18.74275		****	****	****			
8	BSE1	18.79775			****	****	****		
2	ChevARQ	19.01725				****	****	****	
1	RomVRQ	19.03988				****	****	****	
10	Swale	19.18100					****	****	****
11	SSBP/1	19.33863						****	****
9	ChevVRQ	19.57675							****

Here the groups 1-3 containing BSE-in-sheep samples do not overlap with groups 4-7 that comprise scrapie samples. In other words the two sets of sample means are distinct.

For glycoform ratio analysis the combined density of the signals obtained for all three protein bands is defined as 100% and the contribution of each band calculated as a percentage of the

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total signal. Diglycosylated (top band (H)), monoglycosylated (middle or lower band (L)) unglycosylated (bottom band (U)). These percentages may also be referred to as the relative quantity (RQ).

Table 4: Proportion (%age) of the total signal for the Di-glycosylated Band

		Bonferroni test Homogenous Groups, alpha = .050 Error MS = 6.1943, df = 77					
Cell No.	Sample	Mean	1	2	3	4	5
11	SSBP/1	45.06475	****				
2	ChevARQ	52.81838		****			
3	CH1641	53.35075		****	****		
1	RomVRQ	57.56113			****	****	
9	ChevVRQ	57.67700			****	****	
8	BSE1	58.37587				****	
10	Swale	59.06000				****	
12	BSE2	60.36275				****	
4	ChevBSE1	65.29875					****
6	RomBSE1	65.39400					****
7	RomBSE2	66.53262					****
5	ChevBSE2	66.60538					****

SSBP/1 is significantly lower than all the others. The four BSE-in-sheep sample means, group 5, also form a distinct group of their own.

Table 5: Proportion (%age) of the total signal for the Mono-glycosylated Band

		Bonferroni test Homogenous Groups, alpha = .050 Error MS = 4.0704, df = 77					
Cell No.	Sample	Mean	1	2	3	4	5
7	RomBSE2	21.01325	****				
6	RomBSE1	21.78075	****				
5	ChevBSE2	22.73975	****	****			
4	ChevBSE1	23.23650	****	****			
1	RomVRQ	25.47287		****	****		
10	Swale	25.56825		****	****		
12	BSE2	25.89175		****	****		
9	ChevVRQ	27.36763			****	****	
8	BSE1	27.43637			****	****	
3	CH1641	29.69237				****	****
2	ChevARQ	30.17312				****	****
11	SSBP/1	32.56025					****

The two higher BSE-in-sheep sample means are in group 2 with 'RomVRQ' but are separate from the other scrapie samples.

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Table 6: Proportion (%age) of the total signal for the Un-glycosylated Band

Bonferroni test Homogenous Groups, alpha = .050 Error MS = 1.9198, df = 77										
Cell No.	Sample	Mean	1	2	3	4	5	6	7	8
5	ChevBSE2	10.65487	****							
4	ChevBSE1	11.46500	****	****						
7	RomBSE2	12.45400	****	****	****					
6	RomBSE1	12.82525	****	****	****	****				
12	BSE2	13.74563		****	****	****	****			
8	BSE1	14.18763			****	****	****			
9	ChevVRQ	14.95538				****	****	****		
10	Swale	15.37175					****	****	****	
3	CH1641	16.95688						****	****	
1	RomVRQ	16.96612						****	****	
2	ChevARQ	17.65913							****	
11	SSBP/1	22.37525								****

Group 4 contains both 'RomBSE1' and 'ChevVRQ', which therefore do not differ significantly. Otherwise the BSE-in-sheep and the scrapie sample means are well separated.

Consistency of results: repeatability

Despite variation in the overall gel (replicate) means there appeared to be fairly consistent differences between the samples within each gel. One way of measuring this is by the *repeatability* which is the proportion of the overall variation attributable to the sample differences. This can easily be calculated from the analyses of variance results and gives the values below.

Molecular weights di-glycosylated band: 0.542
 Molecular weights mono-glycosylated band: 0.677
 Molecular weights unglycosylated band: 0.737

 Proportion of signal for the di-glycosylated band: **0.820**
 Proportion of signal for the mono-glycosylated band: 0.681
 Proportion of signal for the unglycosylated band: 0.791

The proportion (%age) of the total signal for the di-glycosylated band has the highest repeatability and therefore may be the best single variable for discriminating between samples.

Within replicate sample variation: box plots

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The variation between samples within replicates can be examined graphically by means of box plots. This has been done in figures 1 and 2 below, which are based on the individual sample values with the replicate mean subtracted. This eliminates the replicate differences and gives a clearer picture of the extent of the overlap of the individual values for different samples. The box covers the central fifty per cent of values and the 'whiskers' indicate the range excluding values considered to be outliers or extreme.

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29th January 2002

Figure 1: Box Plots of Molecular Weights

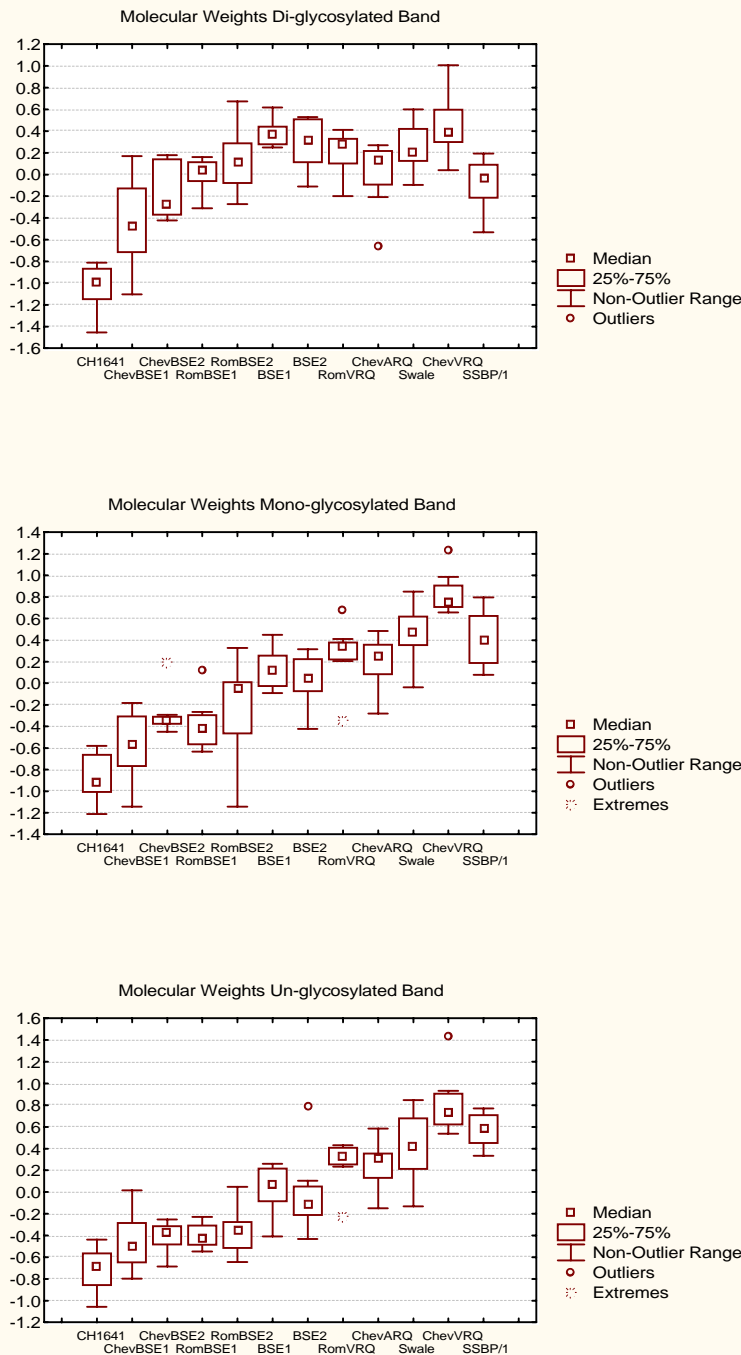
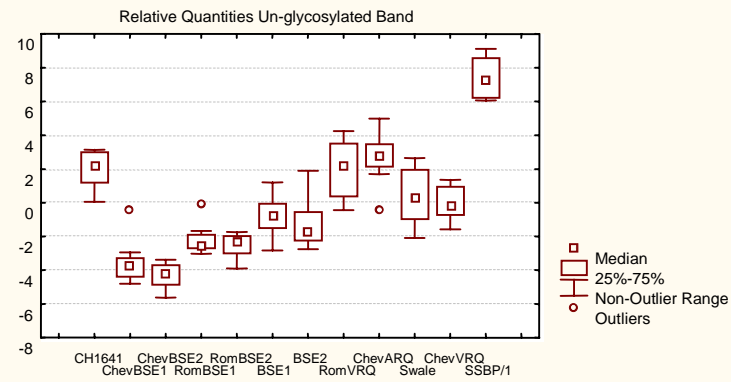
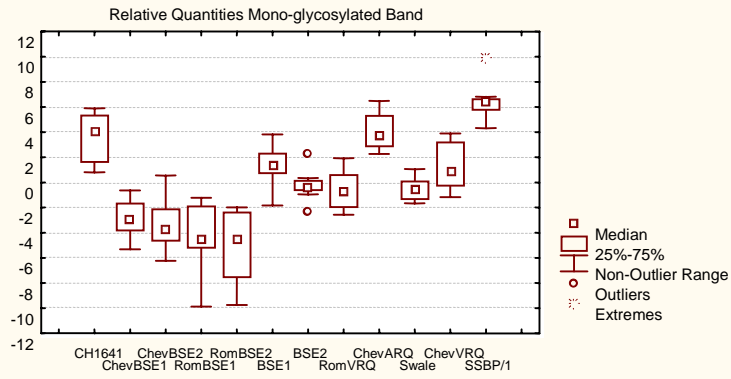
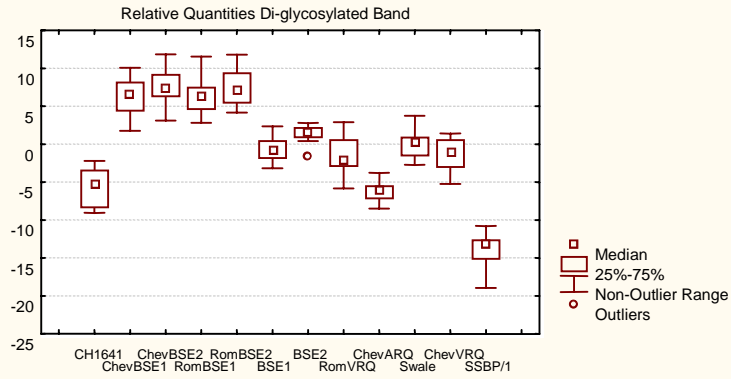


Figure 2: Box Plots of Relative



VALIDATION OF THE HYBRID WESTERN IMMUNOBLOTTING TECHNIQUE FOR TSEs IN SHEEP.

General Summary

- Preliminary results for a panel of ruminant brain tissues that were subjected to a Western immunoblotting technique developed at VLA ('hybrid' technique) are about to be published in *Acta Neuropathologica*. The panel was tested in parallel using two monoclonal antibodies (mAbs) raised against different epitopes of prion protein. Three distinctions could be made between natural sheep scrapie cases and sheep experimentally inoculated with BSE.
 - The molecular weight of the unglycosylated protein band was consistently higher for scrapie samples than that found for brain samples from the Romney and Cheviot breed sheep infected with BSE.
 - The glycoform ratio of the signals obtained for di and monoglycosylated bands also showed differences. The sheep passaged CH1641 scrapie strain gave molecular weights similar to, but not identical to BSE, and a glycoform ratio similar to sheep scrapie cases. The SSBP1 experimental scrapie strain gave molecular weights which were akin to natural scrapie cases but the glycoform ratio was different to that found for all the other samples.
 - When the antibody mAb P4 was substituted for mAb 6H4 in the technique only the natural scrapie samples and SSBP1 gave strong signals. BSE in sheep and the CH1641 strain gave weak reactions and prions from BSE infected cattle could not be detected at all.
- These preliminary results confirmed that the combination of molecular weight and glycoform ratio and differentiation with two specific antibodies, could be used to screen for BSE in the UK sheep flock. Suspicious samples would need to be confirmed as accurate by bioassay and lesion profile analysis in mice inoculated with brain tissue from suspect field cases.
- These results were presented to SEAC and it was recommended that the results under-go statistical analysis and that further work on the reproducibility of the test be carried out. To facilitate this, an outline action plan consisting of four phases was sent to DEFRA. Phase 1 is the statistical analysis of the preliminary results and further evaluation of the technique. Phase 2 is a retrospective examination of brain tissue from approximately 1400 WB-positive sheep brains submitted to VLA since 1st January 1998. Phase 3 is the prospective testing of scrapie submissions by WB and immunohistochemistry and phase 4 will address any further research requirements. The results for Phase 1 are presented in this report.

Summary of Phase 1 Results.

- Statistical analysis of the results from the *Acta Neuropathologica* paper showed that the mean molecular weights for the unglycosylated band in the BSE-in-sheep samples did not overlap natural scrapie sample means and therefore formed distinct groups. The smallest difference between the group members was about 0.6Kd between the BSE infected Romney and the Cheviot ARQ/ARQ scrapie. The greatest difference (1Kd) was between CH1641 and Cheviot ARQ/ARQ. Despite variation in the gel (replicate) means there were consistent differences between samples within each gel. For glycoform ratio analysis the combined density of the signals obtained for all three protein bands is defined as 100% and the contribution of each band calculated as a percentage of the total signal. For SSBP1, the proportion represented by the unglycosylated band was higher, and the diglycosylated band lower, than for all other samples.
- A series of further experiments was set up to assess reproducibility of the VLA hybrid technique in terms of between-gel, within-gel and between-operator variation, to include blind testing, and to assess the effects of autolysis on the results obtained (experiments 1 and 2).

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- The precision of the assay was very satisfactory for the molecular weights, with the coefficients of variation all less than three per cent. Apart from the residual error, the differences in the results between the gels and days made the largest contributions to the variation in the measurements. The differences between operators and readers were mostly small in comparison. With one exception, these differences were less than one per cent of the total variance.
- This was not the case for the glycoform signals obtained from each of the three protein bands. When the signal density for each single band was analysed repeatedly the coefficients of variation exceeded 10%. This was more noticeable for the unglycosylated band. The glycoform ratio measurements were also highly variable. The coefficient of variation for the ratio of the relative quantity of the diglycosylated signal against that of the monoglycosylated signal was 26%. Thus glycoform ratio analysis is a less useful tool than molecular weight analysis for discrimination between BSE and scrapie, except perhaps in instances such as the discrimination between BSE and scrapie strain CH1641.
- Molecular weight is the most promising discriminatory factor but samples should be compared on the same gel to eliminate the between gel variation. To achieve maximum discrimination gels should also include BSE-in-cattle and sheep scrapie material as positive controls.
- Samples of various genotypes were also tested using mAb 6H4. When statistically analysed, no significant differences were found between them for the mean molecular weights. However, the molecular weight results for fresh material were on average consistently higher than for autolysed material, and the differences, though small, were statistically significant for the monoglycosylated and unglycosylated bands. As the effects of autolysis appear to mimic the reduced molecular weight result observed from BSE material, it is important to take account of the time between the death of the animal and the time the sample was taken, and to take note of sample state when analysing the result. This is more likely to be a problem for samples from fallen stock animals than those obtained through the abattoir surveys.
- The hybrid technique is also used to discriminate by utilising the different affinities of mAb 6H4 and mAb P4 to the BSE and scrapie prions. Because of the potential for drift in molecular weight and small molecular weight differences being measured it is essential that the differential antibody binding and molecular weight differences are used for final comparisons of scrapie isolates, alongside the accepted controls. However, although the effect of autolysis on results obtained with mAb 6H4 has been demonstrated, the effects of autolysis on the binding ability of mAb P4 has not been investigated in this study.
- Because we have no data on the behaviour of BSE in most sheep genotypes (only Cheviot AHQ/AHQ and Romney ARQ/ARQ have been tested so far) false negatives cannot yet be ruled out. Also, we have not been able to test any secondary-passaged BSE-in-sheep brain tissue alongside primary transmitted BSE-in-sheep to see whether these findings remain stable.