OPINION ON THE CRL REPORT ON BATCH TESTING OF TSE RAPID TESTS: SAMPLE SELECTION AND TEST SENSITIVITY ISSUES

Opinion of the Scientific Panel on Biological Hazards

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Opinion of the Scientific Panel on Biological Hazards on the CRL report on batch testing of TSE rapid tests: sample selection and test sensitivity issues[1]

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Summary

The European Food Safety Authority (EFSA) and its Scientific Panel on Biological Hazards and the Expert Working Group on Transmissible Spongiform Encephalopathy (TSE) Testing were asked by the European Commission (EC) to evaluate a report of the Community Reference Laboratory (CRL) on batch testing of TSE rapid test kits which highlighted some matters of concern including sample selection and test sensitivity issues. At present, 12 rapid BSE test kits are approved by the EC for the *post mortem* testing of slaughtered cattle in accordance with the TSE Regulation (EC) No 999/2001.

The aim of a "Batch testing" programme is to compare different batches of a particular test kit for consistency of performance. A panel of samples is tested using each new batch of kits produced. The results obtained must fall within pre-determined limits. Batch release testing and /or approval are carried out to varying degrees by Member States. In order to establish a European wide batch testing procedure the CRL has assembled a panel of brain homogenates prepared from BSE positive bovine brain to be used for batch testing purposes. This sample panel was tested by the test manufacturers in their own laboratories using EU approved rapid tests. Most of the tests identified all of the positive samples in the set as positive, with medium to high readings. However, several of the tests failed to detect some of the positive samples, including some strongly positive samples. The CRL prepared a report on the testing and this was communicated to the companies concerned. These companies were given time to respond to the report and their replies were forwarded together with the CRL report to the EFSA for evaluation.

The experts of the Scientific Panel on Biological Hazards (BIOHAZ Panel) reviewed the CRL report on batch testing data and concluded that not all of the nine tests evaluated performed equally. The implications of this are twofold; firstly, the sample panel cannot be used in its current state to provide a batch testing system for all currently approved EU BSE rapid tests, although it is suitable for most of them. Secondly, they also suggest that there are profound differences in performance in terms of robustness, with respect to sample format, displayed by currently approved rapid tests. Consequentially, any observed differences in performance, if real, would be of concern. The observation that aliquots of the same positive sample were found to be highly positive according to some of the approved rapid tests but negative according to others, could be attributable to aspects of the test performance and/or to properties of the sample material tested. These concerns are addressed in a number of recommendations, as formulated in the Opinion.

The BIOHAZ Panel further concluded that these batch testing data do not compromise the previous Institute for Reference Materials and Measurements (IRMM)-EFSA evaluation of rapid BSE tests.

Key Words:

BSE, Bovine Spongiform Encephalopathy, batch testing, rapid BSE test, Regulation (EC) No 999/2001.

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1. Background

According to EU legislation all slaughtered cattle over the age of 30 months have to be tested using one of the EC approved rapid BSE tests (EC, 2001). In addition, a defined number of fallen stock over 24 months of age as well as all emergency slaughtered cattle over 24 months of age have to be tested for BSE with one of the approved rapid tests. Annex X to Regulation (EC) No 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies lists the approved rapid post mortem tests which may be used within the framework of the EU monitoring programmes. The approval of the rapid post mortem tests was based on an EFSA evaluation protocol and its recommendation on the suitability or otherwise of the evaluated tests for inclusion in the EU programme for TSE monitoring. Any subsequent modifications to the test protocol are subject to review and approval by the Community Reference Laboratory for TSEs (CRL) on the basis of evidence submitted by the manufacturer.

Until now batch release testing and /or approval has been carried out to varying degrees by Member States. This varies from full release testing of all batches, to acceptance of the manufacturers release procedure.

In order to provide a unified approach to batch release testing, and eliminate duplication of effort, the European Commission (EC) asked the CRL for TSEs as part of their work programme to establish a coordinated batch testing procedure. The CRL procedure foresees some EU National Reference Laboratories (NRL) being asked to test a panel of BSE homogenate samples comprising negative, weak, medium and high reacting samples with named TSE rapid test kits, as part of a strategic programme to cover all test kits authorised for statutory use within the EU.

2. Terms of reference

The CRL prepared a report on the tests carried out by the test manufacturers in their own laboratories using EU approved rapid tests using a panel of brain homogenates prepared from BSE positive bovine brain and supplied by the CRL. Two approved tests *i.e. Institut Pourquier Speed it test* (IP-test) and the *Enfer test*, recorded low or negative values for the sample set.

Both companies were asked to comment on the CRL report and their comments were also forwarded to EFSA for evaluation together with the CRL report.

According to Article 31 of Regulation (EC) No 178/2002 scientific assistance is requested to assess the CRL report and the observations of the companies concerned, to provide a conclusion on its contents, to compare this information with the data of the JRC-EFSA evaluation reports and, if there are grounds, to review previous opinions recommending these tests for approval.

3. Assessment

3.1. Definition, aim and use of batch testing

The aim of a "Batch testing" programme is to compare different batches of a particular test kit for consistency of performance. A panel of samples is tested on each new batch of kits produced. The results obtained must fall within pre-determined limits. If this occurs, it confirms that the new kit is essentially the same as previous batches and may be used with confidence. If the results are not within limits, it suggests that the kit is different from ones produced previously and should not be used. All TSE rapid test manufacturers undertake "batch testing" for their own kits and release kits that pass the in-house tests. Additionally some countries undertake additional batch testing and some do not.

Each new TSE rapid test kit that enters the market must be authorized for statutory use within the European Union and listed in the TSE Regulation 999/2001. The approval is linked to the particular test protocol used for the original EFSA evaluation study. Any modifications to the protocol are subject to review and approval by the CRL on the basis of evidence submitted by the manufacturer.

The CRL assembled a panel of brain samples prepared from BSE positive bovine brain to use for batch testing purposes. The brains samples are further referred to as "*batch sample panel*" and the method used to prepare the Batch testing sample material is described in the Annex to this opinion**.** The batch sample panel was tested by the test manufacturers in their own laboratories, using EU approved rapid tests.

The aim of the current exercise as carried out by the CRL, was to enable named EU NRLs to test a panel of BSE samples comprising negative, weak, medium and high reacting samples with named TSE rapid test kits, as part of a strategic programme to cover all test kits authorised for statutory use within the EU. This testing would provide a unified approach to batch release testing, and hopefully eliminate duplication of effort. The batch release assessments will be available to all NRLs within the EU thus replacing formal batch release testing required by some individual member states. All EU NRLs and all TSE kit manufacturers have endorsed the strategy.

This exercise is used to compare different batches of a manufacturer's test kit for consistency of performance and not to compare one test kit against another. The strategy was clear on this point and it was the basis upon which the manufacturers agreed to endorse the process.

The report on the batch release procedure identified certain weaknesses which should be addressed and resolved before continuing the batch release testing coordinated by the CRL, as part of the agreed annual CRL working programme.

3.2. Procedure for batch testing at the Community Reference Laboratory (CRL)

3.2.1. Method

Batch sample panels (see annex) were derived from 4 x 1kg pools of macerated bovine brains from BSE cases and negative samples. Details of the individual animals that comprise the pool are not available. The brains were collected from cattle in the UK between August and November 1990. The pools were prepared by processing whole brain material through a mincer with a 10mm extrusion plate. After preparation the brain pools were stored at –20°C until transfer to Veterinary Laboratories Agency (VLA) Weybridge in 1997, after which storage was at -80°C. The pools have been confirmed to be of 100% bovine origin by PCR analysis carried out at Laboratory of the Government Chemist (LGC) at Teddington, UK.

Samples were removed from the freezer and stored at +4°C overnight to defrost. They were then further processed using the CRL's standard method for preparing proficiency test and test evaluation material (see annex). No diluent was added at this homogenisation stage. After homogenisation sample dilutions (one part homogenised whole brain pool: one part negative brain: one part water) were prepared to create a weak sample. The samples later referred to as medium and strong contained no additional negative material. Homogenates were then divided into aliquots, placed into 1.2-1.5g cryotubes and stored at -80°C. At least three samples from each dilution were sent to VLA Newcastle for testing by Bio-Rad TeSeE and to VLA Weybridge for testing by VLA Hybrid Western Blot, to check strength and consistency of result for the aliquot sets. Analysis of the brain pools using the VLA hybrid blot demonstrated a characteristic banding pattern of non-, mono- and di-glycosylated PrP^{sc}.

The first set of batch samples (Round 1) was issued to all EU test kit manufacturers: Idexx (HerdChek), Enfer (Version 2), Bio-Rad (TeSeE), Prionics (LIA, PrioSTRIP and Western Blot), Roboscreen (Beta Prion), Institut Pourquier (Speed'it), Roche (PrionScreen) and Cedi (CEDITECT BSE). Manufacturers were asked to treat the homogenised material as pure tissue, and to test each aliquot twice. Results were returned by all except Cedi and can be seen in Table 1 below. Signal cut-off ratios for Round 1 are presented in Table 1A.

Following analysis of the first set of results, the second set of samples (Round 2) was issued blind to all manufacturers as listed above (except Cedi). All the samples of the second round were tested with two different kit batches. It was suggested by one manufacturer that insufficient tissue was present in the aliquots for one of its tests to work effectively. Therefore all the tests that produced negative results in the first analysis (Institut Pourquier Speed'it and Prionics LIA), or relatively lower positive results for the weak and medium positive samples (Enfer version 2.0) were issued with two sets of samples, one to treat as pure tissue (so would use the amount of homogenate prescribed by their instructions for use), and one to treat as 50% tissue (so would use twice the amount of homogenate prescribed by their instructions for use). Results are given in Table 2 below. Signal cut-off ratios for Round 2 are presented in Table 2A. Two additional positive aliquots were also issued to these manufacturers. They were homogenates that had previously been tested using two of these test kits during the IRMM/EFSA evaluation which resulted in the approval of these test kits and had produced a strong positive reaction. Enfer was also given a set of samples to be used on its Version 3 (TMB assay), which is currently in the approval process. The remaining tests, which had worked effectively the first time, were given two additional negative samples so the sample sets did not appear different between the manufacturers. Results are given in Tables 3 and 3A.

Throughout the exercise, all samples were dispatched to the manufacturers on card-ice to ensure they remained frozen during transit. There were no reports received from the manufacturers of samples being received in a thawed state, and all kits were able to use the samples as expected.

3.2.2. *Results.*

All results in **bold** in the following tables indicate an incorrect diagnosis.

Round 1:

Table 1: **Results for Round 1 of Batch testing suitability assessment:**

Test	Sample Type								*Pos control*	*Neg control*	*Cut-off*
	BSE Negative		Weak BSE Positive		Medium BSE Positive		Strong BSE Positive				
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2			
Bio-Rad (TeSeE)	0.008	0.007	0.655	0.691	2.017	2.012	1.874	1.757	*mean 2.067*	*mean 0.006*	*0.216*
Enfer (Version 2)	1.326	1.42	9.953	7.991	26.44	35.79	37.87	50.01			*5.5*
Idexx (HerdChek)	0.036	0.038	1.805	1.843	2.649	2.886	2.484	2.449	*3.888*	*0.028*	*0.148*
Institut Pourquier (Speed'it)	0.31	0.29	**0.64**	**0.72**	**1.26**	1.46	**1.05**	1.36	*166.33*	*0.07*	*1.3*
Prionics (LIA)	54	44	657	**539**	2802	3376	3134	3305	*High 441'233 RLU's Low 26'020 RLU's*	*35 RLU's*	*590 RLU's*
Prionics (PrioSTRIP)	18	0	352	302	1219	1422	1292	1356	*5606 5266*	*0 and 0*	*60 and 60*
Prionics (Western Blot)	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	*Pos*	*n/a*	*n/a*
Roboscreen (Beta Prion)	0.032	0.038	1.478	1.403	2.962	>3	>3	2.902	*>3*	*0.035*	*>0.2*
Roche (PrionScreen)	0.087		1.713		3.856		3.958		*3.070*	*0.099*	*0.300*

Table 1A: **Signal-Cut-off ratio for Round 1 of Batch testing suitability assessment**

Test	Sample Type					
	Weak BSE Positive		Medium BSE Positive		Strong BSE Positive	
	Result 1	Result 2	Result 1	Result 2	Result 1	Result 2
Bio-Rad (TeSeE)	3.03	3.20	9.34	9.31	8.68	8.13
Enfer (Version 2)	1.81	1.45	4.81	6.51	6.89	9.09
Idexx (HerdChek)	12.20	12.45	17.90	19.50	16.78	16.55
Institut Pourquier (Speed'it)	**0.49**	**0.55**	**0.97**	1.12	**0.81**	1.05
Prionics (LIA)	1.11	**0.91**	4.75	5.72	5.31	5.60
Prionics (PrioSTRIP)	5.87	5.03	20.32	23.70	21.53	22.60
Prionics (Western Blot)	-	-	-	-	-	-
Roboscreen (Beta Prion)	7.39	7.02	14.81	15.00	15.00	14.51
Roche (PrionScreen)	5.71	-	12.85	-	13.19	-

Round 2: Table 2: **Results for Round 2 of Batch testing suitability assessment**

Test	Sample Type													
	BSE Negative		Weak BSE Positive		Medium BSE Positive		Strong BSE Positive		*Pos control*		*Neg control*		*Cut-off*	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*	*Batch 1*	*Batch 2*
Bio-Rad (TeSeE)	0.010	0.018	0.508	0.446	0.751	1.620	1.778	1.692	*2.518*	*2.280*	*0.013*	*0.013*	*0.223*	*0.223*
Enfer (Version 2) (Pure tissue)	-0.105	0.096	**2.315**	**4.560**	15.040	6.228	13.690	19.560	-	-	-	-	*5.5*	*5.5*
Enfer (Version 2) (50% tissue)	-0.037	0.084	**2.495**	**4.512**	19.750	35.090	6.720	17.800	-	-	-	-	*5.5*	*5.5*
Enfer (Version 3) (Pure tissue)	0.0465	-	**0.1305**	-	0.2875	-	0.2815	-	-	-	-	-	*provisionally 0.26*	
Enfer (Version 3) (50% tissue)	0.0385	-	**0.1165**	-	**0.2465**	-	**0.2425**	-	-	-	-	-	*provisionally 0.26*	
Idexx (HerdChek)	0.040	0.030	1.921	2.173	2.341	2.941	2.304	2.887	*3.920*	*3.968*	*0.028*	*0.022*	*0.148*	*0.142*
I-P (Speed'it) (Pure tissue) test 1	0.780	1.074	**1.120**	**2.260**	**1.570**	**1.960**	**1.510**	**2.320**	*258.99*	*221.85*	*0.26*	*0.588*	*3.59*	*4.47*
I-P (Speed'it) (Pure tissue) test 2	0.790	0.095	**1.040**	**1.900**	**1.780**	**1.740**	**1.440**	**2.150**	*258.99*	*221.85*	*0.26*	*0.588*	*3.59*	*4.47*
I-P (Speed'it) (50% tissue) test 1	1.080	0.788	**1.200**	**1.960**	**1.440**	**1.600**	**1.810**	**1.716**	*258.99*	*221.85*	*0.26*	*0.588*	*3.59*	*4.47*
I-P (Speed'it) (50% tissue) test 2	0.740	1.020	**2.090**	**1.656**	**1.650**	**1.500**	**2.110**	**2.410**	*258.99*	*221.85*	*0.26*	*0.588*	*3.59*	*4.47*
Prionics (LIA) (Pure tissue)	54	95	**226**	**360**	1510	2576	1373	1237			*53*	*66*	*701*	*1067*
Prionics (LIA) (50% tissue)	127	228	839	1376	3851	7229	6237	8699			*42*	*28*	*499*	*805*
Prionics (PrioSTRIP)	0	2	242	256	1110	571	1162	696	*5037*	*4799*	*0*	*0*	*60*	*60*
Prionics (Western Blot)	no bands	no bands	3 bands	3 bands	3 bands	3 bands	3 bands	3 bands	-	-	-	-	-	-
Roboscreen (Beta Prion) test 1	0.039	0.044	1.329	1.635	2.452	2.605	3.040	3.041	*4.044*	*3.734*	*0.054*	*0.020*	*0.200*	*0.200*
Roboscreen (Beta Prion) test 2	0.037	0.049	1.559	1.451	3.102	2.457	2.274	2.724	*4.044*	*3.734*	*0.054*	*0.020*	*0.200*	*0.200*
Roche (PrionScreen)	0.1390	0.0840	2.4570	2.0425	3.8810	3.8940	4.0630	4.1200	*3.6305*	*3.3805*	*0.1485*	*0.0740*	*0.3243*	*0.2870*

Note, both Institut Pourquier and Roboscreen tested each aliquot in duplicate, results were expressed on two lines of the above table
As mentioned above, the Enfer, Institute Pourqier and Prionics LIA tested two more strong positives which had previously been used to assess the Enfer version 2 and Institut Pourquier Speed'it in an EFSA evaluation of new rapid tests:

Table 2A: **Signal-Cut-off ratio for Round 2 of Batch testing suitability assessment**

Test	Sample Type					
	Weak BSE Positive		Medium BSE Positive		Strong BSE Positive	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Bio-Rad (TeSeE)	2.28	2.00	3.37	7.26	7.97	7.59
Enfer (Version 2) (Pure tissue)	**0.42**	**0.83**	2.73	1.13	2.49	3.56
Enfer (Version 2) (50% tissue)	**0.45**	**0.82**	3.59	6.38	1.22	3.24
Enfer (Version 3) (Pure tissue)	**0.50**	-	1.11	-	1.08	-
Enfer (Version 3) (50% tissue)	**0.45**	-	**0.95**	-	**0.93**	-
Idexx (HerdChek)	12.98	15.30	15.82	20.71	15.57	20.33
I-P (Speed'it) (Pure tissue) test 1	**0.31**	**0.51**	**0.44**	**0.44**	**0.42**	**0.52**
I-P (Speed'it) (Pure tissue) test 2	**0.29**	**0.43**	**0.50**	**0.39**	**0.40**	**0.48**
I-P (Speed'it) (50% tissue) test 1	**0.33**	**0.44**	**0.40**	**0.36**	**0.50**	**0.38**
I-P (Speed'it) (50% tissue) test 2	**0.58**	**0.37**	**0.46**	**0.34**	**0.59**	**0.54**
Prionics (LIA) (Pure tissue)	**0.32**	**0.34**	2.15	2.41	1.96	1.16
Prionics (LIA) (50% tissue)	1.68	1.71	7.72	8.98	12.50	10.81
Prionics (PrioSTRIP)	4.03	4.27	18.50	9.52	19.37	11.60
Prionics (Western Blot)	-	-	-	-	-	-
Roboscreen (Beta Prion) test 1	6.65	8.18	12.26	13.03	15.20	15.21
Roboscreen (Beta Prion) test 2	7.80	7.26	15.51	12.29	11.37	13.62
Roche (PrionScreen)	7.58	7.12	11.97	13.57	12.53	14.36

Table 3: **Round 2 of Batch Testing suitability assessment, strong positives for luminescent assays and comparison with former results:**

Test	Sample Type								Cut-off for batch testing aliquots	
	BSE positive 1				BSE positive 2					
	Batch testing result		Former result		Batch testing result		Former result			
	Batch 1	Batch 2	Result	Cutoff	Batch 1	Batch 2	Result	Cutoff	Batch 1	Batch 2
Enfer (Version 2) (Pure tissue)	429.2	516.0	849.9	*5.5*	200.8	241.4	513.8	5.5	*5.5*	*5.5*
Enfer (Version 2) (50% tissue)	588.9	740.0	-	-	960.0	1128.0	-	-	*5.5*	*5.5*
Enfer (Version 3) (Pure tissue)	3.6345	-	-	-	2.5035	-	-	-	*Provisionally 0.26*	
Enfer (Version 3) (50% tissue)	2.4375	-	-	-	2.1225	-	-	-	*Provisionally 0.26*	
I-P (Speed'it) (Pure tissue) test 1	20.98	17.76	1077.70*	*330.80*	6.14	8.20	717.60*	330.80	*3.59*	*4.47*
I-P (Speed'it) (Pure tissue) test 2	20.65	17.39	5945.10*	*199.70*	5.75	7.59	3336.90*	199.70	*3.59*	*4.47*
I-P (Speed'it) (50% tissue) test 1	29.82	34.18	-	-	7.70	9.68	-	-	*3.59*	*4.47*
I-P (Speed'it) (50% tissue) test 2	27.85	42.56	-	-	7.02	8.54	-	-	*3.59*	*4.47*
Prionics (LIA) (Pure tissue)	16715	50862	-	-	5310	10754	-	-	*701*	*1067*
Prionics (LIA) (50% tissue)	106944	160373	-	-	28399	45258	-	-	*499*	*805*

Institut Pourquier also reported the following results from IRMM samples run on the same plate as the batch testing samples, these used the IRMM generic homogenate and not one made with "Speed'it" specific buffer:

- Batch 1: BSE IRMM = 7.35, Scrapie IRMM = 89.54
- Batch 2: BSE IRMM = 6.73, Scrapie IRMM = 92.7

* The "former results" for the IP test were obtained during testing of different aliquots of the same samples during the IRMM/EFSA evaluation. One set of tests was carried out at AFSSA in Lyon and the other set of tests was carried out at VLA Newcastle. Note the cut-offs for the test are different between the IRMM/AFSSA evaluation and the batch testing. This is because the reader used for the test has been changed, ,resulting in a different scale, in the interim period. This change was validated and shown to produce equivalent results in terms of diagnostic ability.

Table 3A: **Signal-Cut-off ratio for Round 2 of Batch Testing suitability assessment, strong positives for luminescent assays and comparison with former results**

Test	Sample Type					
	BSE positive 1			BSE positive 2		
	Batch testing		Former test	Batch testing		Former test
	Batch 1	Batch 2		Batch 1	Batch 2	
Enfer (Version 2) (Pure tissue)	78.04	93.82	154.53	36.51	43.89	93.42
Enfer (Version 2) (50% tissue)	107.07	134.55	-	174.55	205.09	-
Enfer (Version 3) (Pure tissue)	13.98	-	-	9.63	-	-
Enfer (Version 3) (50% tissue)	9.38	-	-	8.16	-	-
I-P (Speed'it) (Pure tissue) test 1	5.84	3.97	3.26	1.71	1.83	2.17
I-P (Speed'it) (Pure tissue) test 2	5.75	3.89	29.77	1.60	1.70	16.71
I-P (Speed'it) (50% tissue) test 1	8.31	7.65	-	2.14	2.17	-
I-P (Speed'it) (50% tissue) test 2	7.76	9.52	-	1.96	1.91	-
Prionics (LIA) (Pure tissue)	23.84	47.67	-	7.57	10.08	-
Prionics (LIA) (50% tissue)	214.32	199.22	-	56.91	56.22	-

From Table 1 it can be seen that Institute Pourquier and Prionics LIA both fail to identify all the positive samples and that the Enfer version 2 has results lying relatively close to the cut-off value for this test. Table 1a shows the signal to cut-off ratios for all tests and samples, with any result below 1.0 being negative. All tests, with exception of Institute Pourquier Speed'it, Prionics LIA correctly identified the positive samples. However, the results of the Enfer version2 were close to the cut-off values. More than one manufacturer made the observation that the middle and strong homogenates are very close together and that the strong may not be the most positive under testing conditions. This is accepted, as only crude estimations were made during production, using different pots of this pooled material. Nevertheless, for each between-test comparison on a sample, all aliquots were derived from the same original pool.

Table 2 shows that the problem of the relatively low results close to the cut off value for the medium and the positive samples continues for three tests (Institut Pourquier Speed'it, Prionics LIA and Enfer version 2), when samples are treated as 100% tissue. However, the Prionics LIA records much higher results when samples are treated as 50% homogenates. Thus we cannot use the sample panel for two of these tests (Institut Pourquier Speed'it, and Enfer version 2), even at double sample weight. Again, Table 2a shows signal to cut-off ratios, with results below 1.0 being negative.

Table 3 shows that the 3 luminescent assays all detect strong reactors. This is consistent with results from the initial EFSA evaluation. However, values are lower, especially when Table 3a signal to cut-off values are taken into account.

3.2.3. Discussion.

The results raise some concerns about the robustness of some tests with respect to sample format, because some of the samples that are intended for use for batch testing are classified as negative or very low positive by some tests. The same sample sets are recorded as strong positives in other tests, so much so that additional dilutions are needed in order to be on the log part of the curve and provide a proper assessment of these tests. Estimates of prevalence would be biased for those tests, as a lower analytical and diagnostic sensitivity could lead to an underestimation of the true BSE prevalence as the risk of false negatives increases.

As the first set of results was unexpected, the exercise was repeated. As one manufacturer thought that its test was particularly susceptible to protein concentration, it was requested that an additional set of samples be treated as 50% homogenates rather than whole brain, and thus twice as much sample was added into the testing process. This was done for all tests that were giving problem results.

The batch testing protocol as set up by the CRL is not intended to assess and compare analytical sensitivity. The results, however, indicated some practical problems: the panel of samples can be used for batch testing for all the tests that have participated in the trial apart from IP, Prionics and Enfer**.** However, it is not practical to make separate sample sets for individual tests.

While it could be argued that the method of preparation (homogenisation) of the sample sets affected some tests and not others, the method (see annex) used for this study is similar to that used to prepare the 200 positive samples used in the IRMM/EFSA evaluation, which resulted in the approval of some (including IP and Enfer) test methods in 2004. Additionally, all CRL-prepared proficiency test samples are prepared in a similar way, as homogenates, being a practical method of preparing identical samples for proficiency testing, and hitherto no significant problems have been identified. Consequently, this factor alone does not appear to explain the differences observed here.

The most likely explanation is that there is an observed difference in analytical sensitivity between the tests. In the EFSA evaluation studies (EFSA, 2005) one of these two tests (IP) was at the lower end of performance in terms of analytical sensitivity (1/64), but the Enfer test had a detection limit of >1/200. At that time the dilution series were not extended further than a dilution of 1/200, so the full information on relative analytical sensitivity of the different test methods is not available.

This may be important, particularly against a background in which an increasing number of healthy cattle that do not show clinical signs of BSE are being tested. If infected with BSE, such animals are likely to have low levels of abnormal PrP present in their brain tissue. The apparent difference in diagnostic sensitivity observed here, which resulted in aliquots of the same sample being identified as highly positive by some of the approved rapid tests but which were classified as negative by other tests, may arise in the field, and eventually result in different detection rates, depending upon the particular rapid test in use. It is acknowledged that there are other issues (such as sampling, the level of laboratory training, etc.) that influence the results obtained during routine testing. However, note that these differences in sensitivity is observed here when the tests were conducted under the best conditions, *i.e.* in the manufacturers' hands.

3.3 Replies from the companies

Comments were received from two companies of which the test was named in the CRL report. It concerns comments by:

- Murex Biotech Ltd covering for both Murex Biotech Ltd. (subsidiary of Abbott) and Enfer Scientific Ltd. Ref letter SANCO/E2/KVD/mb/D(2006) 521127 (13 November 2006) with letter of company in annex.
- Institut Pourquier. SANCO/E2/MP/mtd/D(2006) 521208 (27 November 2006) with letter of company in annex. Ref letter 0611184 (14 November 2006).

Comments addressed the panel of samples, the batch release testing system and the analytical sensitivity.

Comments on the panel of samples were made with respect to the provenance of the starting material for the dilutions and, more in particular the effect of homogenisation and the prolonged storage of these samples are addressed, potentially influencing the native structure of the abnormal prion and thus influencing the signal. Further concerns were expressed in terms of lack of details on storage time and temperature of these samples.

On the batch release testing system it was stressed that the purpose of this testing is to ensure consistency between batches, not to compare manufacturers. In this respect it was suggested that several panels or even individual panels per manufactures were supplied. With respect to the latter, the company suggested that each manufacturer was to supply its own panel of samples for their own assay.

Comments on the analytical sensitivity relate to the assumption that low performance on diluted samples equates to low diagnostic sensitivity.

Although different in approach and length, both companies concluded that the starting material was unsuitable and inappropriate for its intended use and that the intention to use a single batch release panel for all assays has been shown not to be a practical proposition.

It is underlined that the comments as expressed by the companies to the CRL report were duly taken on board and addressed in the current Opinion of the EFSA BIOHAZ panel.

3.4. Requirements to be considered for future Batch testing:

A protocol for batch testing including proper definition and number of samples is available but needs to be refined for use under the following conditions:

i. Manufactures need to provide clear specifications on the conditions under which samples should be prepared and stored (*i.e.* commutability).

ii. Test manufacturers can either use a panel of samples supplied by the CRL or their own panel of samples. In the latter case, the panel of samples should be prepared under CRL supervision. In addition, an external sample will be used by the NRL/CRL to control this panel of samples. This panel should be supplied in sufficient quantity to allow this comparison to be completed over time.

iii. If the CRL panel of samples is not used, the test manufacturer should provide the CRL with proper documentation, attesting that the samples used comply with the conditions defined above.

4. Conclusions

1. The BIOHAZ Panel reviewed the Community Reference Laboratory (CRL) batch testing data and concluded that not all of the nine tests evaluated, performed equally. In particular, the inability of several of these tests to detect some clearly positive batch testing samples raises questions regarding the robustness of these tests with respect to sample format.
2. The BIOHAZ Panel concluded that these batch testing data do not compromise the previous IRMM/EFSA evaluation of rapid BSE tests. The BioHaz Panel noted that in previous evaluations, tests were approved for their ability to confirm a case of BSE in a clinically suspect animal and for use to estimate the prevalence of BSE as a clinical disease in a cattle population.
3. The BIOHAZ Panel recognized that an inferior analytical sensitivity could lead to inferior diagnostic sensitivity which could then lead to an underestimation of the true BSE prevalence, specifically in the context of a declining BSE epidemic, where the majority of the animals will be in a pre-clinical status of infection.

5. Recommendations

1. The biological basis of the differences in analytical sensitivity of BSE field tests is un-quantified and requires to be clarified.
2. The BIOHAZ panel recommends further assessment of currently approved tests to detect potential changes in performance with time or between batches, which may affect the usefulness in determining disease/infection prevalence especially in the frame of the present decreasing BSE prevalence.
3. The BIOHAZ Panel recommends the need for rigorous Quality Assurance (QA) of rapid BSE tests. Batch testing is an important part of this QA and the Panel supports the fact that the CRL, as a matter of urgency, is to finalize an appropriate protocol in accordance with the guidelines expressed in this opinion.

6. Documents Provided to EFSA

Letter with the ref. D(2006)KVD/khk/521097 from the European Commission, Health & Consumer Protection Directorate-General (DG SANCO) requesting scientific assistance to assess the CRL report on the Batch Testing of TSE rapid test kits. With Annex: CRL report: Batch testing of TSE rapid test kits: sample selection and test sensitivity issues

7. References

EFSA (2005) Scientific report of the European Food Safety Authority on the evaluation of rapid *post mortem* tests intended for small ruminants. *The EFSA Journal*, **31**; 1-17.

EU (2001) Regulation (EC) No 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. *Official Journal of the European Union* **147**: 1- 40.

Annex: Preparation of Batch Release Samples

Sample origin, pre –treatment and storage

Sample panels were derived from 4 x 1kg pools of macerated bovine brains from BSE cases and negative samples. Details of the individual animals that comprise the pools are not available. The brains were collected from cattle in the UK between August and November 1990. The pools were prepared by processing whole brain material through a mincer with a 10mm extrusion plate, after preparation the brain pools were stored at –20°C until transfer to Veterinary Laboratories Agency (VLA) Weybridge in 1997, after which storage was at -80°C. The pools have been confirmed to be of 100% bovine origin by PCR analysis carried out at Laboratory of the Government Chemist (LGC) at Teddington, UK.

Processing of batch release material

Samples were removed from the freezer and stored at +4°C overnight to defrost. They were then further homogenised using the CRL's standard method for preparing proficiency test and test evaluation material, however no diluent was added at this homogenisation stage.

The brain material was weighed and further disrupted using a hand held blender, with metal blades. Three cycles of 30 seconds, operating at room temperature and low speed were used. After disruption with the blender, the sample was assessed visually by the operator and, if required because lumps of tissue were visible, further cycles of tissue disruption were carried out. The disrupted sample was mixed on a vortex mixer for one minute to remove any surplus air bubbles and to ensure that the sample was thoroughly mixed. Details of the preparation were entered on the sample management database

After homogenisation, a dilution (1part positive whole brain material [brain pool pot 3] was prepared by adding two parts negative brain homogenate (this was 1 part brain, 1 part water) to create a weak sample. The samples referred to as medium (brain pool pot 2) and strong (brain pool pot 4) contained no additional negative material or water. The disrupted material was then divided into aliquots, placed into barcoded 1.2-1.5g cryotubes and stored at -80°C.

At least three samples from each preparation (negative, weak, medium and strong) were tested by Bio-Rad TeSeE and by VLA Hybrid Western Blot assays, to check strength and consistency of results for the aliquot sets. Analysis of the batch testing material using the VLA hybrid Western Blot demonstrated a characteristic banding pattern of non-, mono- and di-glycosylated PrP^{sc} . An example of the results obtained for the strong positive sample (pot 4) is shown in tables 1 and 2, and in figures 1 and 2.

Table 1. **Example of Bio-Rad TeSeE results for strong positive batch testing sample**

Aliquot reference	Sample	Test	Result	Ratio OD/cut-off
CBH00605	pot 4	BioRad TeSeE	1.411	6.53
CBH00606	pot 4	BioRad TeSeE	1.396	6.46
CBH00881	pot 4	BioRad TeSeE	1.427	6.61
CBH00882	pot 4	BioRad TeSeE	1.256	5.81
CBH01159	pot 4	BioRad TeSeE	1.209	5.60
CBH01160	pot 4	BioRad TeSeE	1.441	6.67

Table 2. **Example of VLA Hybrid Western Blot Results for strong positive batch testing sample (Date received: 15/08/2005; Date tested 25 and 26 / 05/2005)**

Ref No.	TMB ID	Results
CBH 00607	J4293	Positive
CBH 00608	J4294	Positive
CBH 00883	J4295	Positive
CBH 00884	J4296	Positive
CBH 01157	J4297	Positive
CBH 01158	J4298	Positive

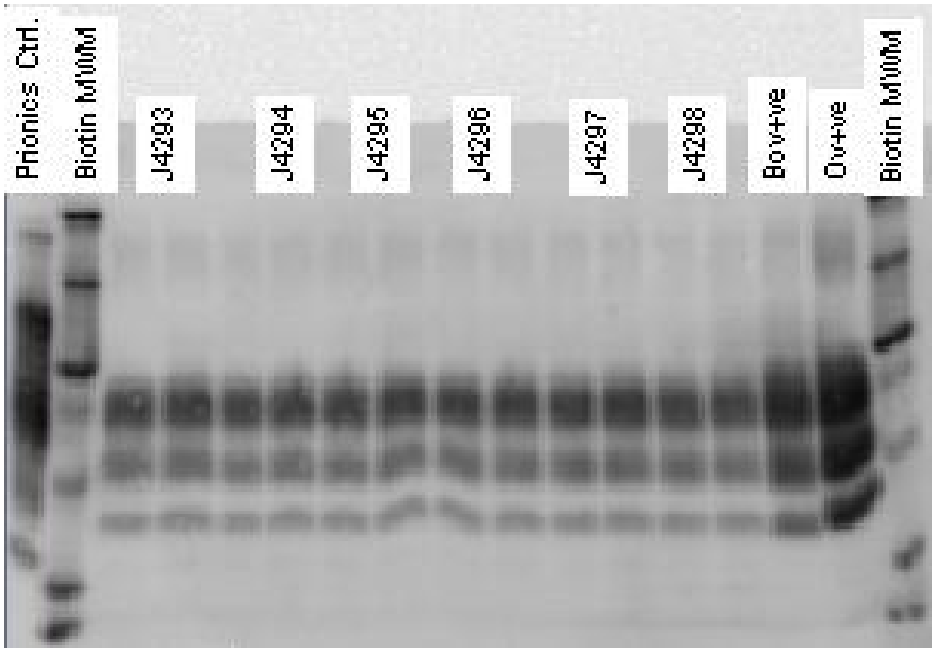

Figure 1: **VLA Hybrid Western Blot assay using mAB 6H4, 10 min reading (D634, 26/08/2005)**

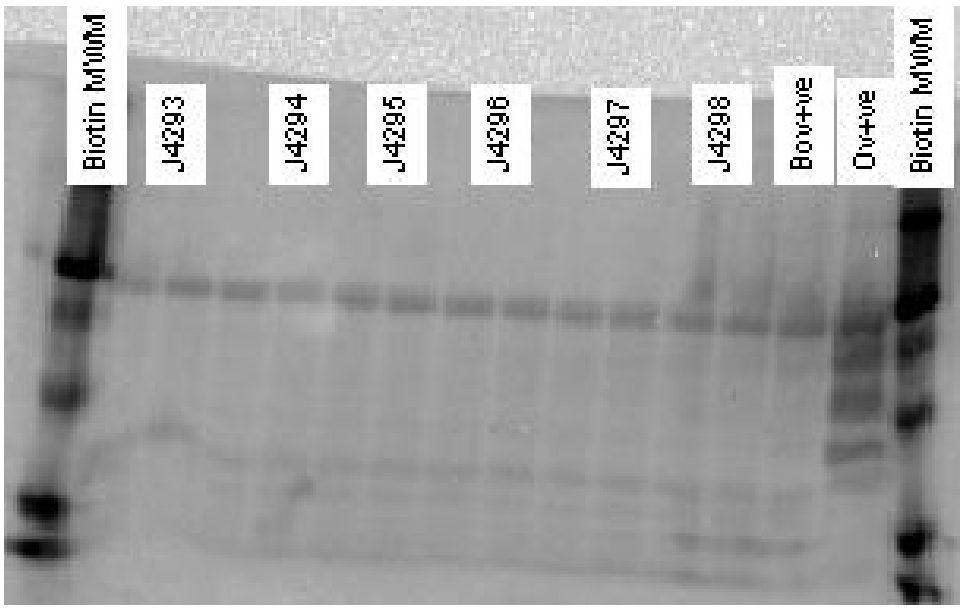

Figure 2: **VLA Hybrid Western Blot assay using mAB P4, 10 min reading (D634, 26/08/2005)**