

A case of atypical scrapie/Nor98 in a sheep from New Zealand

Abnormal prion protein consistent with atypical scrapie/Nor98 has been detected in the brain of a New Zealand sheep that was part of a large consignment of brains sent to Europe for use as negative control material.

Nature of atypical scrapie/Nor98

Accumulated scientific knowledge to date has demonstrated that atypical scrapie, also known as Nor98, is clinically, pathologically, biochemically and epidemiologically different from classical scrapie⁽¹⁾.

The majority of atypical scrapie/Nor98 cases have been identified in clinically normal sheep sampled at slaughter⁽¹⁻³⁾. Cases have been reported from Norway, Sweden, Finland, the UK, Germany, France, Portugal, Belgium, the Netherlands, Ireland, Denmark⁽⁴⁾, the United States⁽⁵⁾ and Canada⁽⁶⁾. The apparent prevalence of the cases in the European Union (EU) Member States is very low, for example for the UK it is less than 0.1%. Active surveillance programmes have led to the detection of atypical scrapie/Nor98 cases even in the absence of any case of classical scrapie in sheep in Portugal, Denmark, Sweden and Finland⁽⁴⁾, and in the Falkland Islands⁽⁷⁾, the first region in the Southern Hemisphere to report such a case.

Atypical scrapie/Nor98 appears to be a non-contagious, sporadic degenerative condition of older sheep. A spontaneous aetiology, possibly with a genetic determinant, environmental influences and metabolic factors, has been suggested in a number of studies based on epidemiological evidence^(2-4, 8). Contributing to this suggestion is the observation that atypical scrapie/Nor98 cases have been detected by active surveillance programmes, wherever such programmes have been initiated⁽⁴⁾. Most cases which have been detected in a flock have been the only case and this lead to early speculation that the condition is not naturally infectious⁽¹⁻³⁾. Experimental oral transmission to sheep has been unsuccessful, to date. Experimental oral transmission into standard mice and into bank voles also failed⁽¹⁾. Only experimental intracerebral transmission to transgenic mice expressing the ovine^(9, 10) or porcine⁽¹¹⁾ prion gene and experimental intracerebral challenge in sheep were successful⁽¹²⁾.

Unlike classical scrapie, abnormal prion protein has not been detected in peripheral tissues of sheep affected by atypical scrapie/Nor98^(1, 4).

The OIE Terrestrial Animal Health Code chapter on scrapie specifically excludes atypical scrapie/Nor98 and explicitly states that the condition is unrelated to 'classical' scrapie, is probably not contagious and may be a spontaneously occurring condition⁽¹³⁾.

It seems that wherever scrapie surveillance in sheep raising countries is intensified and suitable tests are used, atypical scrapie/Nor98 is found. Recently, three cases of atypical scrapie/Nor98 were reported in a UK research sheep flock derived from animals of New Zealand origin ⁽¹⁴⁾. Subsequent investigations in the UK did not find any evidence that the condition was either acquired in the UK or introduced from New Zealand. Thus the discovery of an atypical scrapie/Nor98 case in an otherwise healthy New Zealand sheep is not entirely unexpected ⁽¹⁵⁾; and reinforces the view that atypical scrapie/Nor 98 occurs spontaneously or naturally in very small numbers of older sheep in every population.

Background

New Zealand, as a country free from scrapie and bovine spongiform encephalopathy (BSE), has been a regular supplier of the EU for ruminant brain tissues as negative control materials for evaluations of rapid tests for the detection of BSE and scrapie ⁽¹⁵⁾. Over the last decade, brain tissues from a total of 4900 animals, including cattle, sheep and goats, have been supplied to the EU.

In 2008, a consignment of 800 sheep and 150 goat hind brains, and 200 sheep whole brains were sent to the Institute for Reference Materials and Measurements (IRMM) in Belgium. Prior to dispatch all brainstem material was screened with the Bio-Rad ELISA, a rapid TSE test. All brain stem samples tested negative. The institute organises the rapid TSE test evaluations in Europe on behalf of the European Food Safety Authority (EFSA).

In July 2009, the brain stem and cerebellum from one whole brain (sheep #1512) from the 2008 consignment were combined into a macerate (a homogenate of equal amounts of tissue and water). This macerate tested positive in one European laboratory using the Bio-Rad ELISA.

These test results were reported to MAF BNZ's Investigation and Diagnostic Centre (IDC), Upper Hutt, in September 2009 and led to the investigation into this case.

Laboratory Investigation and Results

Overview of results

While both classical scrapie and BSE were ruled out with the brain stem tissues available, the extent of the laboratory investigation for the confirmation of atypical scrapie/Nor98 was hindered due to the limited availability of appropriate brain tissues, such as fresh and formalin-fixed cerebellum, from sheep #1512.

Figure 1 shows the brain tissues that remained left at IRMM in September 2009 after the brain stem and the cerebellum had been removed for the production of the macerate (grey square A, not to scale). Figure 2 shows the geographical locations and movements of tissues between various institutions and the numerous tests that have been performed over time. Bold numbers in square brackets refer to Table 1, where the chronology of testing and test results are listed.

Testing at IDC prior to shipping to IRMM

A small slice of brain stem posterior to the obex, indicated by arrow and #1 in Figure 1, and a slice from the cerebellum had been retained as fresh tissue reference material at IDC. A small slice of the brain stem anterior to the obex, fixed in formalin, indicated by arrow and #2 in Figure 1, had also been retained in New Zealand.

In April 2008, prior to the shipping of the brains, the slice of posterior brain stem had been tested in the Bio-Rad ELISA at IDC with negative results (see Table 1 and Figure 2). This ruled out classical scrapie and BSE being present in this brain. According to evaluation data for the Bio-Rad ELISA ⁽¹⁶⁾, abnormal atypical scrapie/Nor98 prion protein should have been detected in the brain stem if it had been present. Hence there was no suspicion of abnormal prion at this time in samples from sheep #1512.

Brain processing and initial testing in Europe

At VLA, under contract from IRMM, all available brain stem and cerebellum were used to make 20g of tissue into macerate. This macerate tested positive in the Bio-Rad ELISA at VLA in July 2009. Subsequent testing of brain stem material organized by IRMM at another European laboratory using the IDEXX ELISA, gave negative results in August 2009.

IDC was informed of these European test results on 2 September 2009.

Testing at IDC during investigation phase, post 2nd September 2009

Initial results were confusing: the Bio-Rad ELISA presented a strong positive reaction with the cerebellar tissue but a negative reaction with the brain stem. The Prionics western blot assays showed only a weak staining pattern for both tissues that appeared to be non-specific.

Subsequent testing in the Bio-Rad western blot (WB) showed a clear staining pattern characteristic of atypical scrapie/Nor98 in the cerebellum and negative results for the brain stem (Figure 3). These results corroborated the previous Bio-Rad ELISA results.

At the request of IDC, half of the brain material still available at IRMM, Belgium (Figure 1), was returned to New Zealand at the end of September 2009. From this the parts of the brain that could harbor abnormal atypical scrapie/Nor98 prion protein, such as frontal cerebral cortex (FCC), parietal cerebral cortex (PCC), and caudal cerebral cortex (CCC), as well as spinal cord as control material, were tested at IDC in the Bio-Rad ELISA and Bio-Rad WB with negative results.

Results at IDC from both Bio-Rad ELISA and Bio-Rad WB indicated abnormal prion protein indicative of atypical scrapie/Nor98 only within the cerebellum and not in other relevant parts of the brain. In view of this, it was decided to request confirmatory IHC testing on fixed tissue at VLA, Weybridge, UK (an OIE Reference Laboratory for BSE and scrapie), to confirm the presence of abnormal prion proteins indicative of atypical scrapie/Nor98 in sheep #1512. IDC also requested that the Bio-Rad WB be conducted on remaining macerate of combined brain stem and cerebellum. Unfortunately there was no fixed or fresh cerebellum available to be used at VLA.

Testing at VLA

The formalin-fixed brain stem from anterior to the obex that had been retained in New Zealand was sent to VLA. In addition, a slice of the frozen midbrain tissue, held at IRMM, Belgium, was also sent to VLA and half of it was fixed in formalin. All fixed tissues were subjected to histopathology/IHC and each returned negative results.

The Bio-Rad WB on brain stem/cerebellum macerate and midbrain tissue showed very weak staining patterns suggestive of atypical scrapie/Nor98. The IDEXX ELISA performed on the macerate gave a negative result.

On 7 October 2009, VLA reported their results for sheep brain #1512 as negative for classical scrapie but inconclusive for atypical scrapie/Nor98. At this time the lack of cerebellar tissue prevented any final conclusion on the presence or absence of atypical scrapie/Nor98.

VLA made the following recommendations on how to proceed, which were agreed to by IDC: to test the macerate in the analytically more sensitive test methods, scrapie-associated fibrils (SAF) western blot and sodium phosphotungstate (NaPTA) western blot.

On 22 October 2009, results for the confirmatory western blots were reported to IDC. The SAF western blot on the macerate showed a strong banding pattern. The banding profile for sample #1512 matched closely that to the known positive atypical scrapie control and overall, was considered positive for atypical scrapie/Nor98 at VLA, UK. A similar pattern of weak staining was also found in the mid brain sample.

The NaPTA western blot run was unsuccessful as the negative control samples exhibited residual normal prion protein due to incomplete digestion with proteinase K and therefore, these results were not used for the final diagnosis.

Discussion

Test results obtained for various brain tissues from sheep #1512 confirmed that neither classical scrapie nor BSE were present. The results suggested that abnormal atypical scrapie/Nor98 prion protein was present mostly in the cerebellum but this could not be confirmed by IHC because intact tissue from the cerebellum was not available. Conclusions have had to rely on various types of confirmatory western blots performed on homogenized tissues.

There are distinct differences in the staining patterns in western blots for the abnormal prion proteins of classical scrapie and atypical scrapie/Nor98. After proteinase K treatment, all classical scrapie strains, and also BSE prions, show a typical triplet pattern comprising the di-, mono-, and unglycosylated bands migrating between 18 and 30 kDa^(17, 18). Western blot patterns for atypical scrapie/Nor98 include multiple protein bands of different sizes, with a characteristic unglycosylated low molecular weight band below 15 kDa^(1, 19-21). Such a pattern was seen in the cerebellum and in the macerate for sheep brain #1512 and as a very weak pattern in the midbrain.

In the majority of atypical scrapie/Nor98 cases reported to date, abnormal prion protein is found in the cerebellar and cerebral cortex, and in the brain stem at lower concentration^(1, 22, 23). In some cases abnormal prion protein is minimal or totally absent from the cerebellum, while in other cases the cerebellum is the only brain area harbouring this prion isoform^(1, 22). Sheep #1512 appears to be a rare case where abnormal prion protein was present almost exclusively in the cerebellum.

The contrasting results for the macerate using the Bio-Rad ELISA and IDEXX ELISA were unexpected. The IDEXX ELISA is one of three rapid screening tests that have been recommended by EFSA for atypical scrapie/Nor98 testing on brain stem and cerebellum⁽¹⁶⁾. Therefore, a positive result for the macerate which contained the cerebellum would have been expected. A recent publication reporting on the first six atypical scrapie/Nor98 cases in the USA showed at least two cases in which brain stem and cerebellum were positive in the Bio-Rad ELISA but negative in the IDEXX ELISA⁽⁵⁾. This suggests that the diagnostic sensitivity of the IDEXX ELISA may be less than initially published results implied.

In summary, the SAF western blot results on the brain stem/cerebellum macerate at VLA, UK, confirmed the previous results obtained on the cerebellum in the Bio-Rad WB at IDC, New Zealand. Sheep #1512 represents a unique case of atypical scrapie/Nor98 in which the abnormal prion protein resided almost exclusively in the cerebellum with low

concentration detected in mid brain and none in the brain stem, and where one of the major screening tests failed to detect its presence.

A more comprehensive scientific publication on this case is planned to follow in due course and will include more detailed western blot results and prion genotyping results of sheep #1512.

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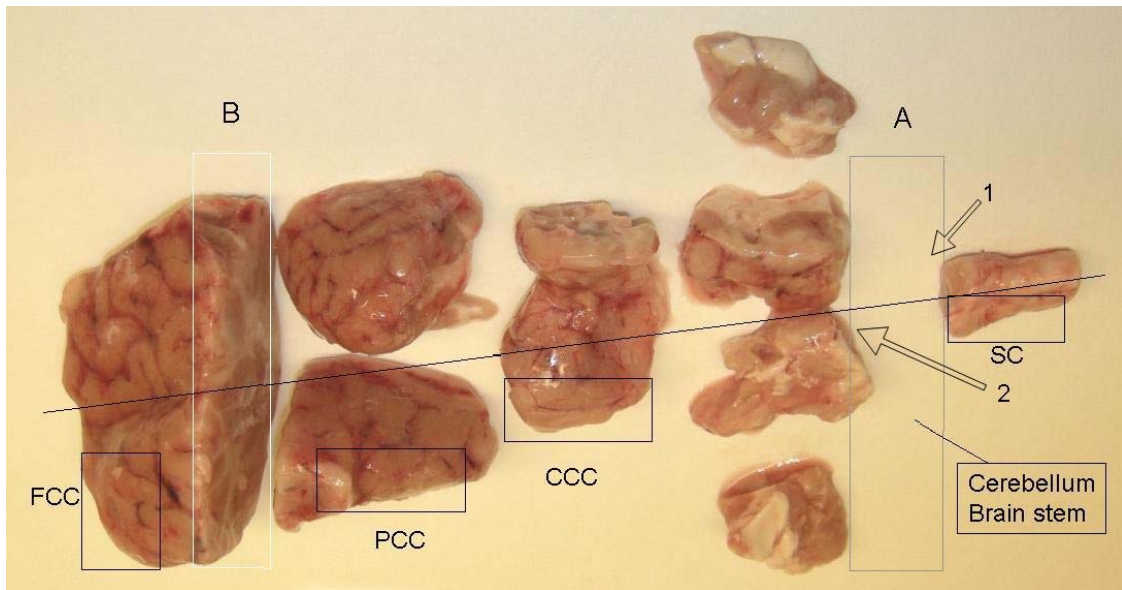


Figure 1: Brain tissues from sheep 1512

Brain tissues from sheep 1512 are shown as they were available at IRMM, Belgium, without the brain stem and cerebellum. Grey Area (A) shows the position of the brainstem. A longitudinal half of the brain tissues (indicated by black line) was returned to New Zealand, where the Bio-Rad western blot and ELISA testing was performed on the tissues as labelled: SC = spinal cord; CCC = caudal cerebral cortex; PCC = parietal cerebral cortex; FCC = frontal cerebral cortex. White area B shows the transverse cut of the midbrain that had been removed for further testing at VLA, UK. Arrows numbered 1 and 2 show where the brain stem tissue slices that had been retained in New Zealand had been removed.

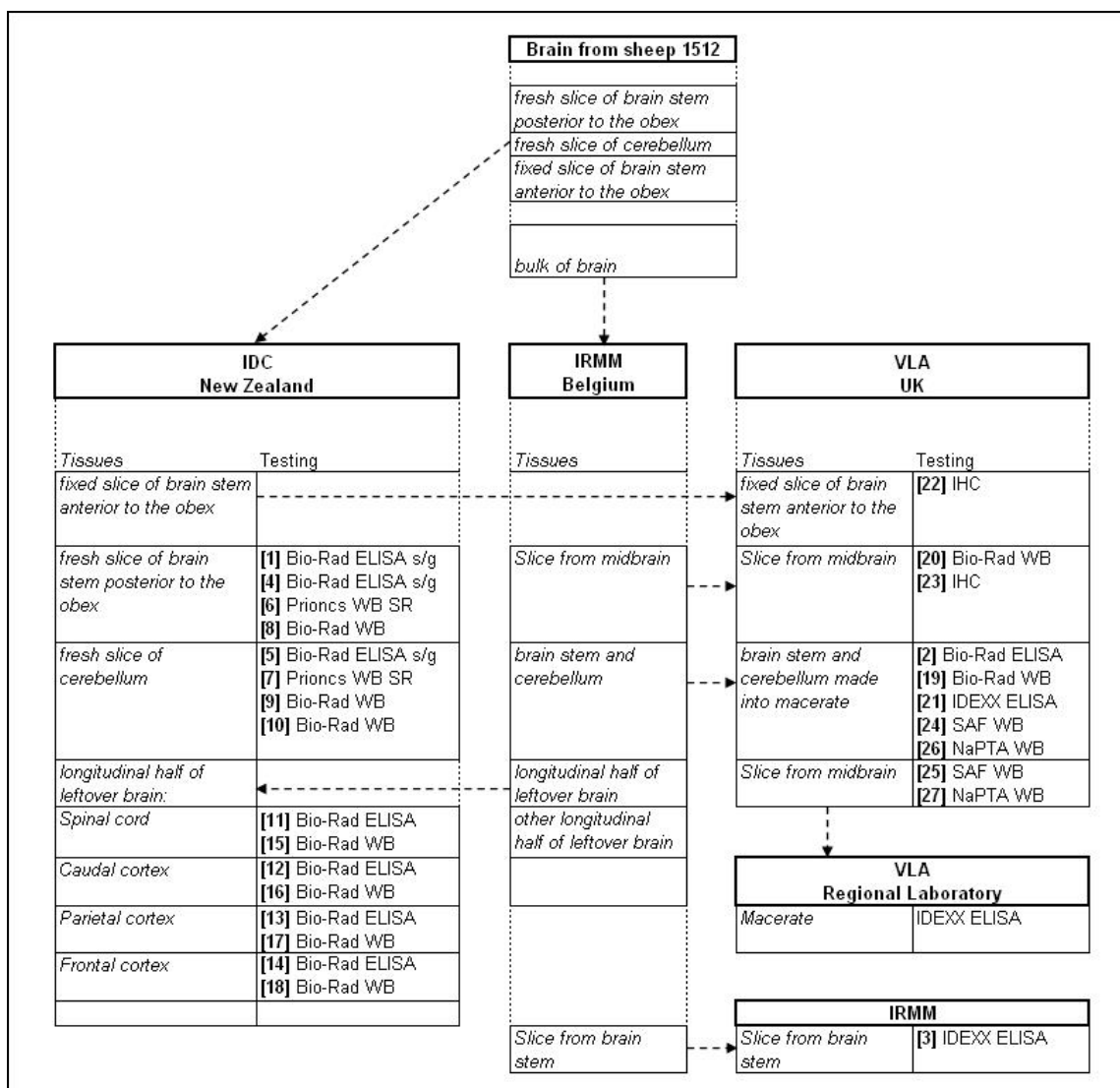


Figure 2: Flow diagram showing use and movement of brain tissues from sheep 1512 and testing performed at various institutions.

Note: Tissue movements and testing are not in chronological order. Bold numbers in square brackets refer to numbers in Table 1, where the chronological order of testing and test results are listed.

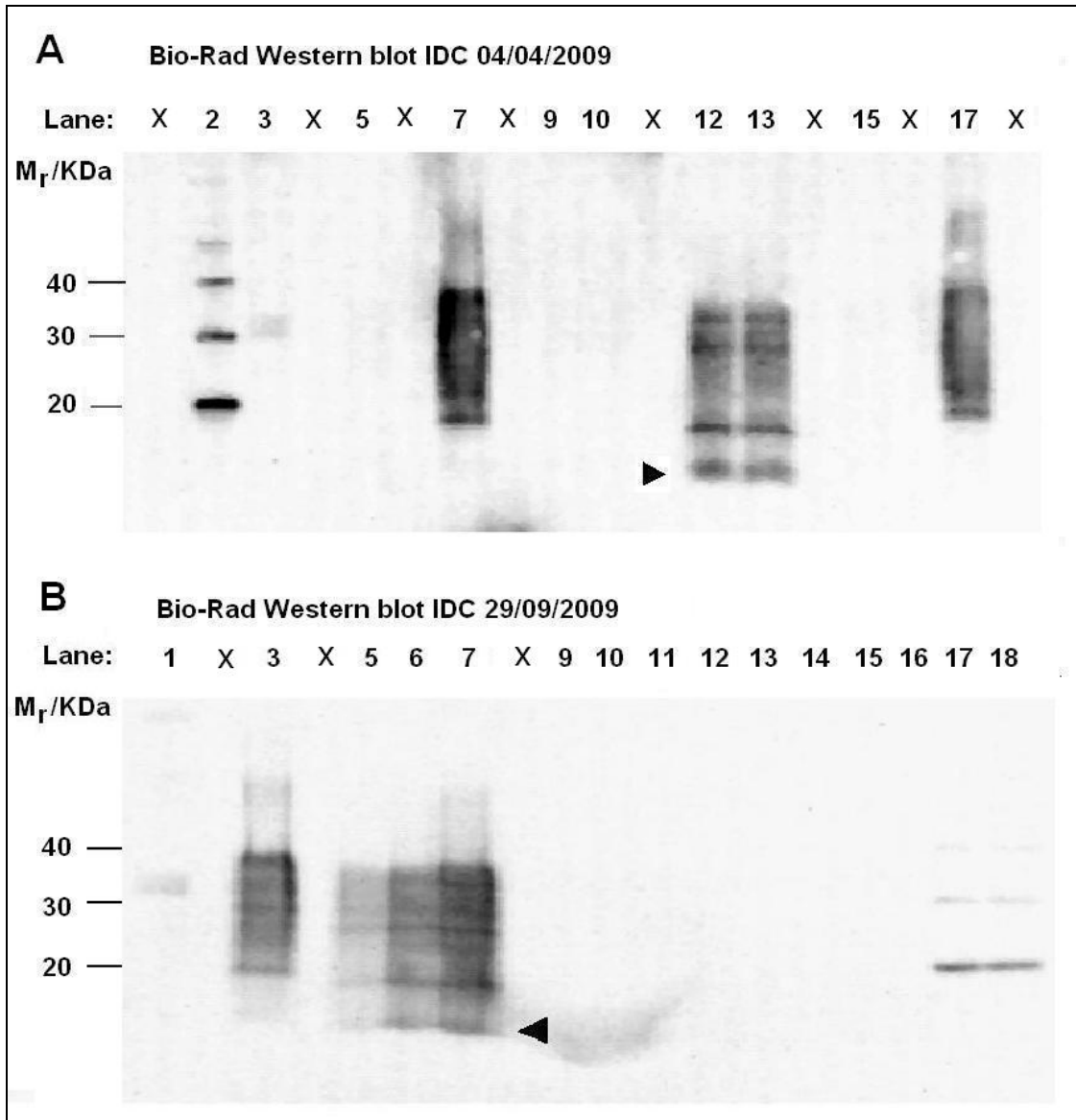


Figure 3: Bio-Rad western blots performed at IDC on various tissues from sheep 1512 on 4th and 29th September 2009

Relative molecular mass (M_r) for MagicMark markers are shown on the left. Arrowhead indicated low M_r in atypical scrapie/Nor98 cases. The smudge in blot B on bottom of lanes 9, 10 and 11 is non-specific staining caused by stray light of the imaging system.

A - Western blot 4/9/2009

X marks empty lanes;
Lane 2 MagicMark marker;

Lane 3 Kaleidoscope marker;
Lanes 5 and 15 atypical scrapie control (failed);
Lanes 7 and 17 classical scrapie control;
Lanes 9 and 10 brain stem sample posterior to obex from sheep 1512;
Lanes 12 and 13 cerebellum sample from sheep 1512.

B - Western blot 29/9/2009

X marks empty lanes.

Lane 1 Kaleidoscope marker;
Lane 3 classical scrapie control;
Lanes 5, 6 and 7 atypical scrapie control;
Lanes 9 and 10 spinal cord from sheep 1512;
Lanes 11 and 12 caudal cerebral cortex from sheep 1512;
Lanes 13 and 14 parietal cerebral cortex from sheep 1512;
Lanes 15 and 16 frontal cerebral cortex from sheep 1512;
Lanes 17 and 18 MagicMark marker.

Table 1: Chronology of testing performed on various brain tissues from sheep 1512

Ref. No	Test method	Tissue tested	Result	Time tested	Testing laboratory
[1]	Bio-Rad ELISA	posterior brain stem	Negative	10/04/2008	IDC AHL
[2]	Bio-Rad ELISA	brain stem/ cerebellum macerate	Positive	8/7/ 2009	VLA, UK
[3]	IDEXX ELISA	brain stem slice	Negative	9/08/2009	IRMM contract laboratory
Positive European results reported by IRMM to IDC on 2/9/2009					
[4]	Bio-Rad ELISA	brain stem posterior to obex	Negative	3/9/2009	IDC AHL
[5]	Bio-Rad ELISA	cerebellum	Strong positive	3/9/2009	IDC AHL
[6]	Prionics WB SR	brain stem posterior to obex	Inconclusive; Weak banding pattern	3/9/2009	IDC AHL
[7]	Prionics WB SR	cerebellum	Inconclusive; Weak banding pattern	3/9/2009	IDC AHL
[8]	Bio-Rad WB	brain stem posterior to obex	negative	4/9/2009	IDC AHL
[9]	Bio-Rad WB	Cerebellum	Strong banding pattern inconclusive because of failure of atypical scrapie control; Suggestive of atypical scrapie/Nor98	4/9/2009	IDC AHL
[10]	Bio-Rad WB	Cerebellum	Strong banding pattern highly suggestive of atypical scrapie/Nor98	11/9/2009	IDC AHL
[11]	Bio-Rad ELISA	Spinal cord	negative	29/9/2009	IDC AHL
[12]	Bio-Rad ELISA	Caudal cerebral cortex	negative	29/9/2009	IDC AHL
[13]	Bio-Rad ELISA	Parietal cerebral cortex	negative	29/9/2009	IDC AHL
[14]	Bio-Rad ELISA	Frontal cerebral cortex	negative	29/9/2009	IDC AHL
[15]	Bio-Rad WB	Spinal cord	negative	29/9/2009	IDC AHL
[16]	Bio-Rad WB	Caudal cerebral cortex	negative	29/9/2009	IDC AHL
[17]	Bio-Rad WB	Parietal cerebral cortex	negative	29/9/2009	IDC AHL
[18]	Bio-Rad WB	Frontal cerebral	negative	29/9/2009	IDC AHL

		cortex			
[19]	Bio-Rad WB	brain stem/ cerebellum macerate	Weak staining, inconclusive	7/10/2009	VLA, UK
[20]	Bio-Rad WB	Midbrain cortex	Weak staining, inconclusive	7/10/2009	VLA, UK
[21]	IDEXX ELISA	brain stem/ cerebellum macerate	negative	7/10/2009	VLA, UK
[22]	IHC*	Brain stem	negative	7/10/2009	VLA, UK
[23]	IHC*	Midbrain	negative	7/10/2009	VLA, UK
[24]	SAF Western blot	brain stem/ cerebellum macerate	Clear staining consistent with atypical scrapie/Nor98.	21/10/2009	VLA, UK
[25]	SAF Western blot	Mid brain	Weak staining consistent with atypical scrapie/Nor98.	21/10/2009	VLA, UK
Case sheep 1512 confirmed as atypical scrapie/Nor98 21 October 2009					
[26]	NaPTA Western blot	brain stem/ cerebellum macerate	Test run failed.	21/10/2009	VLA, UK
[27]	NaPTA Western blot	Mid brain	Test run failed.	21/10/2009	VLA, UK

Ref No. = Numbers in bold square brackets refer to the numbers in Figure 2.

WB = western blot; IHC = immuno histochemistry.

*While brain stem and midbrain gave negative results in IHC, the overall results for IHC was inconclusive at this stage, since no cerebellum tissue was available for testing.