



UNCONFIRMED ELISA POSITIVE SAMPLES IN THE UK NATIONAL SCRAPIE SURVEY OF CULL SHEEP

Issue

1. Following the publication by Defra of the results of scrapie surveillance in sheep in Great Britain, an Expert Panel group convened on 17th September 2003 to discuss unconfirmed results that had arisen from the survey of cull sheep. The Group subsequently issued a statement detailing the discussion and conclusions of the panel meeting.
2. The Chair of the group Professor Chris Bostock, will report the main findings of the panel to the Committee. A copy of the group's statement is attached at Annex 1.

Background

3. A summary of the results of scrapie surveillance in sheep in Great Britain (Jan 2002-March 2003) was published on Thursday 11th September 2003. This report showed that 28 sheep were positive by the Bio-Rad Platelia assay but these results could not be confirmed by OIE approved confirmatory methods (immunohistochemistry). At Defra's and the FSA's request, an expert group was asked to advise on the scientific basis and significance of these inconsistent results.

Advice sought from the committee

Members are asked to:

- Note the statement issued by the expert group.
- Comment on the findings and conclusions reached by the panel.

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Expert Panel Group conclusions from meeting on Wednesday 17th September 2003

Background

1. A summary of the results of scrapie surveillance in sheep in Great Britain between January 2002-March 2003 was published by Defra on 11 September 2003 <http://www.defra.gov.uk/animalh/bse/bse-publications/bse-publications-index.html>. This included an abattoir survey of sheep greater than 18 months of age, a survey of fallen stock, an anonymous postal survey of breeding flocks and the results of routine notifications of clinically suspect cases of scrapie.
2. In the abattoir survey, 29,201 (out of a total of 50,630) sheep were tested using the Bio-Rad Platelia ELISA test on brainstem. Twenty-four of these sheep tested positive for a TSE by the Bio-Rad Platelia ELISA and were confirmed positive by immunohistochemistry (IHC), an OIE approved diagnostic method. There were an additional 28 animals where the results were inconsistent, testing positive by the Bio-Rad Platelia ELISA but negative by immunohistochemistry, making their TSE status unclassifiable. Some of the unclassified brainstem samples were from sheep with PrP genotypes not normally associated with scrapie.

Expert Group

3. Following requests from FSA and Defra a Group comprising SEAC members and experts in protein chemistry and TSEs convened on 17th September with the following terms of reference:
 - To review the results of the Scrapie Surveillance report and preliminary research conducted by the VLA to investigate possible reasons for the lack of correlation between the Bio-Rad screening test and the OIE confirmatory tests used in this survey.
 - To recommend and prioritise further work that should be undertaken to help resolve outstanding analytical issues identified by the expert panel.
 - To advise on research needed to clarify the biological significance of these findings when they are shown to be analytically interpretable.
3. The Expert Group comprised the following members:

- Professor Chris Bostock (Chair) – SEAC
 - Dr Martin Groschup – Centre for Virus Diseases of Animals, Germany
 - Dr Thierry Baron – Agence Française de Sécurité Sanitaire des Aliments, Lyons, France
 - Professor Guy Dodson – Head of Division of Protein Structure, National Institute for Medical Research, Mill Hill, UK
 - Dr Jean-Philippe Deslys – Head of the Prion Protein Research Group, Commissariat à l’Energie Atomique, France
 - Professor Robin Carrell – Department of Haematology, University of Cambridge, UK. SEAC member
 - Dr Jiri Safar – Institute for Neurodegenerative diseases, Department of Neurology, University of California, USA. SEAC member
5. The expert group received a presentation from VLA scientists on the results of preliminary research carried out to characterise the samples which gave inconsistent results from the scrapie survey. The group also received presentations on related research from members of the expert group.
 6. The group agreed that the research data on the unclassified samples were preliminary and, at this stage, difficult to interpret usefully. The details of some test reagents were not available to VLA scientists or the expert group (for commercial or pre-publication reasons). Thus it was not possible to examine, and hence attempt to interpret, the basis for the different results obtained with the Bio-Rad Platelia ELISA and the OIE tests used in the scrapie surveillance report. Members of the expert group agreed that, in their experience and depending on test conditions, it was likely that the Bio-Rad ELISA and Bio-Rad Western blot assays may be more sensitive in detecting the marker of TSE infection than other rapid tests currently used in Europe. In addition to potential differences in the inherent sensitivity of the various tests, it was noted that the test result may also depend on the region of the brain sampled for testing. Finally the group was concerned that premature disclosure of such preliminary research could lead to misguided speculation and compromise subsequent scientific publication about the biological significance of the data.
 7. The group agreed that, as a first step, the key issue was to investigate the scientific basis for the lack of correlation between the different tests. It was agreed that immediate discussions should be held with the companies involved to make it possible to achieve this.
 8. Experts attending the group from other countries reported that similar, but not identical, inconsistent results had been observed in France and Germany, with samples testing positive by ELISA but initially negative by Prionics Western blotting. However these inconsistent results had all eventually been resolved by confirmation of positivity by IHC, and/or, in the case of the German sheep, by SAF immunoblotting. These differences in results may reflect differential sensitivities of the tests used in the various laboratories, the possibility that non-identical target sites may have been sampled or real variations between strains of TSEs. It was noted that the PrP genotypes of sheep whose samples initially produced unclassified survey results in France differed from those of sheep producing unclassified survey results in the UK; in particular the absence of unclassified samples from sheep with the ARR/ARR PrP genotype. There has been one sample considered as unclassified from an ARR/ARR sheep in Germany. The group agreed

that significant further work was required on the analytical and diagnostic sensitivity of the various tests with exchange of antibodies, reagents, samples and reference materials between the Community and National Reference Laboratories performing the tests.

9. The issue of PrP and the resistance of its various forms to Proteinase K (PK) digestion was considered. Apart from the Conformation-Dependent Immunoassay, all of the current EU-approved rapid tests rely on the relative resistance of the disease-associated form, PrP^{Sc}, to PK digestion. It is known that different patterns of resulting PK-resistant PrP-derived molecules can be seen on Western blots, depending on the infecting strain of TSE, the digestion conditions, and the properties of the antibodies used. The group noted two distinct properties of the PrP in the UK unclassified samples:

(i) its degradation was slower than that of PrP^C but faster than that of disease-associated PrP^{Sc},

(ii) in contrast to PrP^{Sc} from naturally and experimentally infected animals the characteristic 27-30 kDa PK-resistant core was not formed.

However the group agreed that in view of the paucity of the data it was not possible to assess the implications of these observations. In comparing the behaviour of PrP in the unclassified samples with the properties of PrP in various experimental models of TSEs some members considered that the results may be indicative of an unusual association of PrP^C molecules rather than reflecting a PrP^{Sc}-like conformational change. Other members suggested that the findings were compatible with a slower, but not necessarily infective, propagation of PrP^{Sc}-like PrP in genomes such as ARR/ARR that are resistant to the development of clinical disease.

10. Members referred to experimental data that showed the ratio of PK-sensitive to PK-resistant PrP differed between different strains in the same host. Members highlighted that PK sensitivity of PrP can be influenced by many factors, including PrP conformation, concentration and analytical test conditions. The tissue and cellular localisation of PrP may also influence its resistance to PK. Therefore these issues should be explored assuming that a full spectrum of PK sensitivity is possible for the PrP protein. Further work is required on material from unclassified samples to characterise the tissue and cellular distribution of accumulated PrP, its ability to aggregate and the kinetics of its digestion by PK.
11. The possible influence of genetic differences, both in the PrP gene itself and other non-PrP genes, on the expression and form of the PrP protein was raised. The group agreed it was possible that polymorphisms within the PrP protein could affect its conformation and cellular location, which in turn could influence its digestion by PK. However they thought it was unlikely that polymorphisms within the PrP coding sequence could be the sole explanation for the different sensitivities between the different tests. The possibility exists that polymorphisms in the PrP gene promoter, or in non-PrP genes, could affect the level and tissue distribution of PrP^C expression. The group agreed that sporadic and/or somatic mutations, which if present in the promoter region of the gene could lead to increased expression of the prion protein, could not be excluded at this stage. Over-expression could influence the processing of PrP^C by cells or the kinetics of any conversion reaction from PrP^C to PrP^{Sc}. This was exemplified in the transgenic experiments that showed that the

level of expression of PrP^C can be a more important influence on the PK resistance than the genotype. The group agreed it would be a useful and straightforward experiment to sequence the promoter and exons of the PrP gene from the unclassified samples. They acknowledged that sequencing of the entire PrP gene was more technically challenging and time consuming.

12. The group also recognised that the rapid tests had been developed and validated for the detection of BSE in cattle where there is a single strain and where no PrP polymorphisms that appear to affect disease progression have been identified. It was important to recognise that the use of these tests in sheep needed to take account of the many different strains of scrapie and the several PrP polymorphisms that have been shown to have widely differing effects on disease outcome. The expert group considered further work was required to characterise PrP patterns from different TSE strains in different PrP genotypes of sheep and at different times during the incubation period.
13. The group had been asked to consider whether the unclassified results could represent unrecognised sub- or pre-clinical BSE in the sampled sheep. The group reviewed the limited published data for primary (cow-to-sheep) and secondary (sheep-to-sheep) experimental transmissions of BSE to sheep of different genotypes. The characteristic BSE-like profile of PK-resistant PrP fragments in Western blots was observed in primary oral infections of ARQ/ARQ sheep and primary intracerebral infections of AHQ/AHQ and ARR/ARR sheep. This BSE pattern was maintained following intravenous sheep-to-sheep transmission in ARQ/ARQ sheep. By contrast, the preliminary data on the unclassified samples indicated that PrP in these samples was more susceptible to PK digestion than PrP^{Sc} from experimentally BSE-infected sheep and that PK digestion of unclassified samples did not yield PK-resistant PrP fragments characteristic of BSE-like profiles. Brain samples from sheep both pre-clinically and clinically infected with BSE stain positively by IHC unlike the unclassified UK samples. The group agreed that the hypothesis that the unclassified samples represent sub- or pre-clinical BSE in sheep could not be ruled out on the basis of the available evidence, but noted that the PrP patterns on Western blots and following IHC of experimental BSE in sheep (as well as those of natural and experimental BSE in cattle) did not resemble those seen in the unclassified samples. Formal confirmation of their dissimilarity would require contemporaneous comparison of the samples using the same analytical procedures.
14. The group agreed that further research into the biochemical and immunochemical properties of the PrP in unclassified samples should help to relate it to the known categories of normal, abnormal and disease-associated PrP, but it would not inform on whether or not it is infectious. This issue could only be addressed by transmission experiments in sheep and mice.
15. Seven of the UK unclassified results were obtained on samples from ARR/ARR sheep, a genotype previously considered to be resistant to scrapie and which is selected for under the National Scrapie Plan (NSP). The expert group agreed that, since the basis for, and significance of, the unclassified results can not be determined on the currently available data, it was not possible to assess what the implications of these results might be for the NSP. Further work is essential to understand the relevance (if any) of these results to the NSP. However the group agreed that, if the research shows that resistant sheep genotypes can harbour sub-

clinical TSE infection, it would be necessary to establish its pathogenesis and to revisit the underlying basis of the NSP.

16. The group was presented with the results of a preliminary “case-control” study of flocks from which 12 of the 28 unclassified sampled sheep had been derived. Factors considered included size, location and recorded presence of scrapie in the flock, membership of the NSP, presence of cattle and other species on the farm and the occurrence of BSE on those farms which also maintained cattle. Although no statistically significant associations had yet been found the group agreed that this was a good approach to identifying potential risk factors associated with unclassified samples and merited further work.

Recommendations of expert group for further research

17. The expert group recommends the exchange of methodologies, antibodies and reagents. There is a need, if possible, to standardise diagnostic protocols across the EU and elsewhere. The group recognised that this falls within the remit of the CRL Expert Group to take forward with the Commission. Where possible, details of reagents, buffers, and antibodies should be made available to collaborators.
18. To establish the biological significance (if any) of the unclassified test results, the group considered it an essential priority that infectivity assays and transmission studies are undertaken in sheep and mice using brain material from animals giving these unclassified samples. Given the long-term nature of this work planning should be initiated without delay.
19. There is a need to build up stocks of key reference materials taken from different genotypes of sheep infected with scrapie and BSE to standardise different tests and determine the affinity constants of the antibodies they use.
20. The expert panel noted that the availability of test material was extremely limited, which greatly restricted the possibilities for further research. This is due to the current SRM restrictions which mean that the heads of sheep are discarded as SRM before test results become available. The group agreed that as much material as possible would need to be made available if research was to continue and recommended that the heads of all sampled sheep should be retained at the abattoir pending the outcome of the diagnostic test. While not helping the analysis of existing unclassified samples, in the future such a measure would secure for further research greater amounts of material from tissue giving inconsistent results on the Bio-Rad Platelia ELISA and tests currently advised by OIE. For any new EU evaluation of rapid tests for use on sheep, the panel recommend inclusion of material from infected sheep of different PrP genotypes. In addition, EFSA should consider the implications of unclassified cases to the evaluation process.
21. It is recommended that the kinetics of PrP digestion by PK be studied on PrP from the unclassified samples, samples from sheep of various genotypes infected with different scrapie strains and BSE and samples from uninfected sheep of various genotypes. It is necessary to know whether extracts from unclassified samples resist PK digestion of PrP and, if the PrP in these samples is fully accessible to protease digestion, it is cleaved at preferential sites indicative of a specific/abnormal structure or is non-specifically degraded.

22. The expert panel recommend sequencing the PrP gene, including the promoter region, for all sheep alleles and for both alleles present in each of the unclassified samples. This will ensure that PrP epitopes recognised by antibodies used in the current tests are not compromised by unsuspected mutational changes and identify any possible common mutation(s) specific to unclassified samples. Mutations in the promoters might indicate that the PrP is being expressed at abnormal levels.
23. The group recommended that, if possible, the diagnostic tests that have given inconsistent results in the abattoir survey should be applied to sheep known to be free of any TSE infection. The group recognised that the availability of such sheep is limited, but, if feasible, such a study might help to establish whether unclassified results are a natural inherent property of a small proportion of sheep or whether they are only found in sheep where a TSE infection can not be absolutely ruled out.
24. The expert group recommended continued epidemiological investigation of the flocks from which the sheep that displayed the unclassified test results had been derived.
25. Finally the group re-considered in turn each of the 14 questions put to the group by the FSA and Defra to confirm that, within the limitations of the available data, each of the questions had been addressed appropriately during the preceding discussions.