EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Scientific Steering Committee

REPORT AND OPINION ON

THE CRITERIA FOR DIAGNOSIS OF CLINICAL AND PRE-CLINICAL TSE DISEASE IN SHEEP AND FOR DIFFERENTIAL BIOCHEMICAL DIAGNOSIS OF TSE AGENT STRAINS

ADOPTED BY

THE SCIENTIFIC STEERING COMMITTEE

AT ITS MEETING OF 13-14 APRIL 2000

OPINION

The European Commission requested the Scientific Steering Committee (SSC) to:

1. Propose criteria for the recognition and differentiation of TSE strains causing scrapie in sheep by molecular/biochemical means.
2. Propose criteria for the diagnosis of scrapie and detection of pre-clinical TSE infection in sheep.
3. Consider the recent publication of Baron et al (1999) and, if appropriate, to amend its Opinion of September 1998 on the *The risk of infection of sheep with BSE* and other opinions related to TSEs in small ruminants.

The SSC requested a working group to address a number of specific questions. These, as well as the complete scientific report of the working group, are attached to the present opinion. On the basis of the report, the SSC concludes as follows:

1. <u>On criteria for the differential biochemical diagnosis of TSE strains</u>:

Regarding criteria for differential biochemical diagnosis of TSE strains it is concluded, that conclusive differential biochemical strain identification is not yet possible and results should be interpreted with caution. In order to make rapid progress, it is recommended that the methodology of biochemical analysis of glycoprotein pattern used in different laboratories be analysed and compared in order to identify all the sources of variation.

2. <u>On criteria for the diagnosis of clinical and pre-clinical TSE disease in sheep</u>:

In respect to the criteria for diagnosis of clinical and pre-clinical TSE disease in sheep, microscopic examination of the brain was not considered to guarantee the confirmation of diagnosis in all clinically suspect cases, especially if the material is autolysed and/or the normal presence of a limited number of vacuoles gives rise to some inconclusive results. The scrapie-associated fibrils (SAF) method, compared to histopathology (HP), misses about 20-25% of the cases and is therefore not recommended unless used on advanced autolysed material where SAF, together with Western blot analysis, are the only applicable tests.

Immunohistochemistry (IHC) and immunoblotting are more sensitive than the HP and SAF methods. There is no conclusive evidence as to whether IHC is superior in sensitivity to immunoblotting or vice versa. On one hand, IHC tests, especially the recently improved versions, may be considered superior in sensitivity to immunoblotting when protocols which

include epitope demasking and enhancement stages as well as visualisation systems are used. IHC has also the advantage of showing the microscopic lesions. On the other hand, modern immunoblot systems are now available which may compare to or even top the IHC: the banding pattern of PrP^{Sc} may be much easier to interpret and provide a more specific information than IHC where many different kinds of staining may come up and be misjudged as falsely positive, although in the vast majority of cases the experienced reader would not misjudge artefactual or normal staining.

To cover all possible clinical and pre-clinical TSE cases in sheep the following cases should be evaluated for use in both eradication programmes and optimum surveillance: all clinically suspect cases, all animals found dead above 1 year of age (FD) showing spongiform encephalopathy by classical histopathology (HP) alone, all clinically suspect cases of prion diseases, FD positive cases found by immunohistochemistry or Western blot/Dot Blot (WB/DB) alone and all those cases of pre-clinical TSE or FD scoring positive with an IHC or WB/DB test, as well as cases with and positive histopathology towards the end of the incubation period. SAF results should always be confirmed by an additional test (IHC, HP or WB/DB).

The SSC recommends that presently available laboratory tests and techniques for the diagnosis of scrapie should be compared for their sensitivity and specificity. Furthermore, rapid diagnostic tests, similar to the ones recently evaluated by the European Commission for BSE in cattle, should be developed and evaluated for scrapie in sheep.

3. <u>On the Baron et al (1999) paper</u>:

Regarding the study of Baron *et al* (1999), it cannot be concluded that the study of Baron *et al* (1999) demonstrates BSE in French sheep. It is therefore not necessary to revise the report of the working group on *The risk of infection of sheep and goats with BSE agent* included in the opinion of the SSC of September 1998 on the same subject. However, the SSC recommends that the continuation and extension of the EU networks on TSE diagnosis in sheep should be funded so that they can be continued beyond the current finish dates.

REPORT OF THE WORKING GROUP

THE QUESTIONS

The European Commission requested the Scientific Steering Committee to:

1. Propose criteria for the recognition and differentiation of TSE strains causing scrapie in sheep by molecular/biochemical means.
2. Propose criteria for the diagnosis of scrapie and detection of pre-clinical TSE infection in sheep.
3. Consider the recent publication of Baron et al (1999) and, if appropriate, to amend its Opinion of September 1998 on the *The risk of infection of sheep with BSE* and other opinions related to TSEs in small ruminants.

BACKGROUND

In a publication by Baron *et al* (1999) "*Similar Signature of the Prion Protein in Natural Sheep Scrapie and Bovine Spongiform Encephalopathy-linked Diseases"* it was suggested that specific molecular features could characterise the protease-resistant prion protein (PrP^{Sc}) detected. Overall results in this publication suggest that scrapie cases with features similar to those of BSE could be found more frequently in sheep than previously described.

This statement should be evaluated in the light of three opinions on aspects of sheep and TSEs: In *The Risk of Infection of Sheep and Goats with Bovine Spongiform Encephalopathy Agent* (adopted by the SSC on 24-25 September 1998) it is stated that high priority should be given to the validation of tests for differential diagnosis of BSE and scrapie. In *Surveillance of TSEs in Sheep and Goat in Relation to the Risk of Infection with Bovine Spongiform Encephalopathy agent and Related Actions to be taken at EU Level* (adopted by SSC 27-28 May 1999) there are a number of proposed actions which include (a) improvement of the methods and speed of large scale application of differential diagnosis of BSE and scrapie on post mortem samples and (b) development of in vivo tests for TSE diagnosis and differential scrapie-BSE diagnosis. In *The Policy of Breeding and Genotyping of Sheep ie the Issue of whether Sheep should be Bred to be Resistant to Scrapie* (adopted by SSC on 22-23 July 1999) it is recommended that strong consideration should be given to the use of appropriate resistant strains of sheep, coupled with the development of and extensive use of validated diagnostic tests. Finally in the UK Spongiform Encephalopathy Advisory Committee (SEAC) report on *Research and Surveillance for TSEs in Sheep* (July 1998) there was strong emphasis that future research should be directed towards diagnostic tests and criteria for diagnosis of clinical and sub-clinical infections.

The SSC therefore requested a working group to address the following questions:

1. <u>On criteria for differential biochemical diagnosis of TSE disease in sheep</u>:

- Western blotting of PrP^{Sc}, or glycoform analysis, reveals protein bands of differing molecular mass which might be used as a strain identifier. Does glycoform pattern vary in different tissue types and band regions? Is the molecular mass of the unglycosylated PrP band significant?

- Is apparent glycoform pattern influenced by the methodology, storage time and/or antibody used?
- What method would the experts recommend for standardisation of glycoform analysis and which tissue should be sampled?
- Differential antibody binding to PrP^{Sc} has been suggested as a method by which strains could be identified. What is known about the sensitivity of this method to its use in different labs with different methods?
- How does differential antibody binding compare with glycoform analysis in the ability to recognise different TSE strains?
- Would the experts recommend that differential antibody binding or glycoform analysis be adopted as a widespread strain typing technique? Are there any other suitable techniques?
- What recommendations would the experts make for standardising strain typing of TSEs by biochemical means throughout the EU?

2. <u>On criteria for diagnosis of clinical and pre-clinical TSE disease in sheep</u>:

- Is there variation in clinical signs in TSE affected sheep through the EU?
- How much variation exists in the number and type of vacuoles detected in TSE affected sheep and which brain areas are affected?
- Is the detection of PrP^{Sc} by immunohistochemistry (IHC) affected by the antibody used and by the details of the technique employed?
- Similarly, is the detection of PrP^{Sc} by Western blotting affected by the antibody and the details of the methodology?
- Is SAF detection reliable? Is it a suitable test for use in all laboratories?
- How sensitive are these methods in comparison with each other?
- How many of the tests (histopathology, IHC, Western blotting, SAF) need to be positive before a TSE diagnosis is accepted? Which test is essential within the minimum number acceptable?

3. <u>On the Baron et al (1999) paper</u>:

- Is it necessary to possibly update/amend the SSC Opinion of September 1998 on the *The risk of infection of sheep with BSE*, in the light of a recent publication of Baron et al (1999) and,
- if appropriate, is it necessary to amend the SSC opinions of 27-28 May 1999 on *Surveillance of TSEs in sheep and goat in relation to the risk of infection with bovine spongiform encephalopathy agent and related actions to be taken at EU level* and of 22-23 July 1999 on *The policy of breeding and genotyping of sheep, i.e., the issue whether sheep should be bred to be resistant to scrapie.*

<u>CONCLUSIONS FROM THE WORKING GROUP</u>

1. <u>On criteria for the differential biochemical diagnosis of TSE strains</u>:

Regarding criteria for differential biochemical diagnosis of TSE strains it is concluded, that conclusive differential biochemical strain identification is not yet possible and results should be interpreted with caution. In order to make rapid progress, the following recommendations are made:

- The methodology of biochemical analysis of glycoprotein pattern used in different laboratories be analysed and compared in order to identify all the sources of variation.
- A workshop should be organised, addressing especially this issue in order to harmonise and standardise the methods and experiments should be proposed to evaluate the possibilities of the biochemical strain typing.

2. On criteria for the diagnosis of clinical and pre-clinical TSE disease in sheep:

In respect to the criteria for diagnosis of clinical and pre-clinical TSE disease in sheep, microscopic examination of the brain was not considered to guarantee the confirmation of diagnosis in all clinically suspect cases, especially if the material is autolysed and/or the normal presence of a limited number of vacuoles gives rise to some inconclusive results. The SAF method, compared to histopathology, misses about 20-25% of the cases and is therefore not recommended unless on advanced autolysed material where SAF, together with Western blot analysis, are the only applicable tests.

There is no conclusive evidence as to whether IHC is superior in sensitivity to immunoblotting or vice versa. On one hand, IHC tests, especially the recently improved versions, may be considered superior in sensitivity to immunoblotting when protocols which include epitope demasking and enhancement stages as well as visualisation systems are used. IHC has also the advantage of showing the microscopic lesions. On the other hand, modern immunoblot systems are now available which may compare to or even top the IHC: the banding pattern of PrP^{Sc} may be much easier to interpret and provide a more specific information than IHC where many different kinds of staining may come up and be misjudged as falsely positive, although in the vast majority of cases the experienced reader would not misjudge artefactual or normal staining. To cover at maximum all possible clinical and pre-clinical TSE cases in sheep the following cases i.e. for eradication purposes, should be included: all clinical suspect cases, all clinical suspect cases or found dead animals above 1 year (FD) showing spongiform encephalopathy with classical histopathology (HP) alone, all clinical suspect cases of prion diseases or FD positive with immunohistochemistry or Western blot/Dot Blot (WB/DB) alone and all cases of pre-clinical TSE or FD scoring positive with histopathology plus one positive IHC or WB/DB test. SAF results should always be confirmed by an additional test (IHC, HP or WB/DB).

Note: the draft non exhaustive matrix for the diagnosis of scrapie in sheep (annex 1), this chart should not be seen as limitative recommendation for the diagnosis of scrapie in the framework of an eradication programme. Eradicating TSE in a location or a country would imply eradicating infection.

3. On the Baron et al (1999) paper:

Regarding the study of Baron *et al* (1999), it cannot be concluded that the study of Baron *et al* (1999) demonstrates BSE in French sheep. It is therefore not necessary to revise the report of the working group on *The risk of infection of sheep and goats with BSE agent* included in the opinion of the SSC of September 1998 on the same subject. However, the Working Group recommends that the continuation and extension of the EU networks on TSE diagnosis in sheep should be funded so that they can be continued beyond the current finish dates. Furthermore, IHC and immunoblotting should be applied in all EU member states and further standardised.

I. TERMS OF REFERENCE

The European Commission requested the Scientific Steering Committee to:

1. Propose criteria for the recognition and differentiation of TSE strains causing scrapie in sheep by molecular/biochemical means;
2. Propose criteria for the diagnosis of scrapie and detection of pre-clinical TSE infection in sheep;
3. Consider the recent publication of Baron et al (1999) and, if appropriate, to amend its Opinion of September 1998 on the *The risk of infection of sheep with BSE* and other opinions related to TSEs in small ruminants.

II. CONTEXT OF THE QUESTIONS

a. There have been three recent working groups on aspects of sheep and TSEs which have produced reports in preparation of opinions of the Scientific Steering Committee. Each has relevance to the current report. In *The Risk of Infection of Sheep and Goats with Bovine Spongiform Encephalopathy Agent* (adopted by the SSC on 24-25 September 1998) it is stated that high priority should be given to the validation of tests for differential diagnosis of BSE and scrapie. In *Surveillance of TSEs in Sheep and Goat in Relation to the Risk of Infection with Bovine Spongiform Encephalopathy agent and Related Actions to be taken at EU Level* (adopted by SSC 27-28 May 1999) there are a number of proposed actions which include (in section 3.4.2) (a) improvement of the methods and speed of large scale application of differential diagnosis of BSE and scrapie on post mortem samples and (b) development of in vivo tests for TSE diagnosis and differential scrapie-BSE diagnosis. In *The Policy of Breeding and Genotyping of Sheep ie the Issue of whether Sheep should be Bred to be Resistant to Scrapie* (adopted by SSC on 22-23 July 1999) it is recommended that strong consideration should be given to the use of appropriate resistant strains of sheep, coupled with the development of and extensive use of validated diagnostic tests. Finally in the UK Spongiform Encephalopathy Advisory Committee (SEAC) report on *Research and Surveillance for TSEs in Sheep* (July 1998) there was strong emphasis that future research should be directed towards diagnostic tests and criteria for diagnosis of clinical and sub-clinical infections.

b. In a recent publication by Baron *et al* (1999) "*Similar Signature of the Prion Protein in Natural Sheep Scrapie and Bovine Spongiform Encephalopathy-linked Diseases*", it was suggested that specific molecular features could characterise the protease-resistant prion protein (PrP^{res}) detected.

   Studies of glycoform patterns of PrP^{Sc} in French cattle with BSE and cheetahs with FSE, as well as in mice experimentally infected by isolates from both species, revealed a consistent molecular signature. Similar studies of 42 isolates from sheep with natural scrapie, from 21 flocks in different regions of France, however, showed three glycoforms indistinguishable from those found in BSE-linked diseases. Moreover, the apparent molecular size of unglycosylated form did not show significant differences among these isolates, and appeared similar to that found in PrP^{Sc} from cattle with BSE. Overall the results reported suggest that the PrP^{Sc} from sheep with scrapie and cattle with BSE share the same molecular signature and this occurs more frequently than previously suspected.

c. In the light of what precedes, the European Commission requested the Scientific Steering Committee to:

1. Propose criteria for the recognition and differentiation of TSE strains causing scrapie in sheep by molecular/biochemical means;
2. Propose criteria for the diagnosis of scrapie and detection of pre-clinical TSE infection in sheep
3. Consider the recent publication of Baron et al (1999) and, if appropriate, to amend its Opinion of September 1998 on the *The risk of infection of sheep with BSE* and other opinions related to TSEs in small ruminants. These are the opinions of 27-28 May 1999 on *Surveillance of TSEs in sheep and goat in relation to the risk of infection with bovine spongiform encephalopathy agent and related actions to be taken at EU level* and of 22-23 July 1999 on *The policy of breeding and genotyping of sheep, i.e. The issue whether sheep should be bred to be resistant to scrapie*.

III. DISCUSSION

Preamble:

The Working Group emphasises that all conclusions and recommendations of this report only apply to sheep and not to cattle.

III.1. Criteria for differential molecular/biochemical aetiological diagnosis of TSE disease in sheep.

Introduction:

There are at least four types of diagnostic parameters, clinical (e.g. scrapie), pathological (e.g. spongiform encephalopathy), biochemical (e.g. prion infection/disease) and aetiological (e.g. disease caused by a specific agent or agent strain such as the BSE agent or scrapie agents). In the case of TSE in small ruminants it is important to be able to distinguish disease, and if possible infection, caused by different aetiological agents. In particular it is important to distinguish between scrapie agents, that are believed only to be animal pathogens, from the BSE agent which is an animal and most likely also a human pathogen, at least when derived from cattle with natural BSE.

At present it is not possible to make an aetiological diagnosis by clinical or pathological means any more than it is possible to make a clinical diagnosis of scrapie or a pathological diagnosis of a TSE by detection of prions. However, it is generally accepted that detection of the conformationally altered form of prion protein, in some circumstances, can be used as a proxy for the presence of TSE infection and for the confirmation of prion disease. To make an aetiological diagnosis, the gold standard method is to identify strains of TSE agent in sheep by a comparative assessment of incubation time and pathological lesion profile of the brains of a panel of 5 strains of inbred mice inoculated with the isolate. This biological system of strain typing is not yet sufficiently developed to the stage where it could be used to investigate large numbers of field isolates from sheep with TSE, despite the development of transgenic mice with shorter incubation periods. The consequence is, at the present time there is an urgent need to be able to rapidly differentiate different agent strains (especially the BSE agent strain) from other strains that may be present in tissues from scrapie infected and

affected sheep. The biological method is currently not appropriate because it takes too long, is expensive, uses animals and is not applicable to large throughputs. Other molecular/biochemical methods could be more appropriate and these are considered below. However, it will be important eventually, to obtain a correlation between the biological strain type determined in mice and the molecular/biochemical strain type before the latter technique is accepted as a proxy for the former.

Alternatively, assays based on the immunochemical characterization of PrP^{Sc} were developed which promised to allow for the investigation of large numbers of field cases of sheep TSE.

1. Western blotting of PrP^{Sc}, or glycoform analysis, reveals protein bands of differing molecular mass which might be used as a strain identifier. Does glycoform pattern vary in different tissue types and in different parts of the same organ. Is the molecular mass of the unglycosylated PrP band significant?
2. Is the apparent glycoform pattern influenced by the methodology, storage time and/or antibody used?
3. What method would the experts recommend for standardisation of glycoform analysis and which tissue should be sampled?
4. Differential antibody binding to PrP^{Sc} has been suggested as a method by which strains could be identified. What is known about the sensitivity of this method to its use in different labs with different methods?
5. How does differential antibody binding compare with glycoform analysis in the ability to recognise different TSE strains? How does this correlate to differences in the proteinase K resistance?
6. Would the experts recommend that the immunochemical characterization of PrP^{Sc} from isolates be adopted as a widespread strain typing technique? Are there any other suitable techniques?
7. What recommendations would the experts make for standardising strain typing of TSEs by biochemical means throughout the EU?

Discussion:

The results of biochemical analysis of glycoprotein patterns of PrP^{Sc} can be influenced by a number of factors such as the different steps involved, the antibodies used and the organs tested. Different patterns may be obtained on brain and tonsils or brain and spleen or brain, spleen and placenta PrP^{Sc} from the same animal (Race *et al*, 1998; Madec *et al*, 2000). However, most results indicate that samples taken from different areas of the brain result in the same pattern using a given test procedure. (Kuczius et al. 1998, Madec *et al*, 2000, Sweeney et al, 2000). However, one study contradicts this statement (Somerville, 1999) where the investigation of three mouse brain areas (cortex, cerebellum and medulla) taken from mice affected by a single strain of scrapie gave different glycosylation patterns. This may have implications for the aetiological diagnosis of sheep scrapie and as long as this is not finally clear, it may be therefore advisable to standardise the brain region being assayed. Using different antibodies or PrP^{Sc} extraction methods on the same samples may result in different Western blot patterns. Moreover, the influence of sheep breed and genotype has not yet been reported but is being studied. So, obtained signatures of PrP^{Sc} should be interpreted with great

care, taking into account all these variables. There is a clear need for standardisation of procedures and antibodies. A number of actions at the EU level, including 1 4 Concerted Action and 3 1 FAIR projects are addressing these issues as well as those in respect to clinical and preclinical diagnosis of TSE in sheep :

- CT 97/3305 (Elsen, France) with 17 partner laboratories, is looking at the genetics of scrapie and strain types of scrapie isolates in many EU countries, in the frame of a European network.
- CT 98/6056 (Lantier, France) is setting up a European scrapie network on epidemiological databases and biological sample banks.
- CT 98/7017 (Elsen, France) is looking for the influence of genes other than PrP in scrapie control.
- CT 98/3651 (Dr S. Done, UK) aims to ensure that tissues and fluid samples of known BSE disease status and quality are available to European scientists for research purposes.
- FAIR PL 98/7021 (Weavers, Ireland) aims to establish a European network for the surveillance of ruminant TSE and the standardisation and harmonisation of the process and criteria for the identification of suspected cases.
- FAIR PL 97/6813 (van Keulen, The Netherlands) aims to study the PrP^{Sc} distribution and kinetics in lymphoid tissues of sheep with natural scrapie and the effects of sheep PrP genotype and scrapie strains.

A range of other methods based on different properties of the PrP are under study but need further validation: a method based on the differences of proteinase K resistance patterns, conformation-correlation-spectroscopy, fluorescence-correlation-spectroscopy (FCS), Fourier transform infrared (FT-IR) spectroscopy, Nuclear Magnetic Resonance (NMR), role of metal ions, etc (Proceedings of Meeting in Tübingen, 23-25/9/1999 on "Characterization and Diagnosis of Prion Diseases in animals and Man").

Also being developed is a new approach to the detection of PrP^{Sc} by immunoblotting formalin-fixed, paraffin-embedded sections of CNS tissue that are collected on to nitrocellulose membranes (Schutz-Schaeffer *et al*, 2000).

Conclusion and recommendations:

Conclusive differential biochemical strain identification is not yet possible and results should be interpreted with caution. However, as much information as possible should be collected about each scrapie sheep isolate. Glycoform analysis adds therefore to the characterization but is on its own not sufficient for strain typing in sheep, nor indeed is any other current test sufficient to ultimately define prion strains.

In order to make rapid progress, the following recommendations are made:

- The methodology of biochemical analysis of glycoprotein pattern used in different laboratories should be analysed and compared in order to identify all the sources of variation.
- A workshop should be organised, addressing especially this issue in order to harmonise and standardise the methods. Experiments should be proposed to evaluate the possibility of biochemical strain typing.

III.2. Criteria for diagnosis of clinical disease and pre-clinical TSE infection in sheep

Preamble: The Working Group emphasises that all conclusions and recommendations of this report apply only to sheep and not to cattle.

Introduction:

There is a need to establish criteria for the clinical diagnosis of scrapie and pre-clinical TSE infection in sheep.

Tests currently available to determine the prevalence of TSEs in sheep were developed for confirmatory diagnosis of clinical cases of scrapie. Such tests are microscopic examination of brain, PrP^{Sc} detection by immunohistochemistry (IHC), PrP^{Sc} detection by western blotting and scrapie associated fibril (SAF) detection by electron microscopy.

Attention is also drawn to the fact that when using very small amounts of proteinase K, intermediate fractions of partially degraded PrP^{C} can appear on Western blot gels and can give misleading results. These intermediate fractions seem to be influenced by the alleles involved (Buschmann et al. 1998).

The question was raised whether cases can reliably be diagnosed by the detection of PrP^{Sc} by immunohistochemistry, immunoblot or other PrP^{Sc} based techniques. Studies using experimental prion strains for infecting conventional and transgenic mice or hamsters (Kuczius and Groschup, 1999) and of diagnostic investigations on field scrapie cases (Madec *et al*, 2000, Manousis et al. 2000) indicate that the extent of PrP^{Sc} accumulation in the CNS may vary between diseased animals and strains independently from the severity of disease (as measured by clinical signs, pathology and/or incubation times). This is also in line with reports from Hadlow *et al*, (1979, 1982) that showed no direct relationship between infectious titers in brain and clinical signs. It was nonetheless agreed that the detection of PrP^{Sc} in clinically affected animal may be used as the most reliable and practicable marker for diagnosing scrapie in sheep.

Ideally, examining serial sections by light microscopy and PrP IHC and adjacent tissue blocks by Western blotting is the best way to proceed with investigations on suspect cases. However, the discrepancy between pathology and PrP biochemistry may be real. Kimberlin and Walker (1988) encompassed this discrepancy in his clinical target area model (CTA) of the pathogenesis of scrapie in the mouse (Kimberlin and Walker, 1988). In the light of this CTA model, discrepancies between PrP^{Sc} and clinical signs are anticipated. More relevant to this present discussion are the studies showing dissociation between PrP^{Sc} (PrP^{res}) and neurodegeneration (Bruce *et al*, 1989; Bruce *et al*, 1994). The identification of other forms of PrP that may cause neurodegeneration as described by Hegde *et al* (1998, 1999) is without any doubt a promising and exciting research, in the field of the relationship between infectious agent, PrP protein and neurodegeneration. The most recently reported work in rodent models of human TSE, suggests that neurodegeneration (spongiform change) may be due to the accummulation, not of PrP^{Sc}, but of a protease-sensitive (at 37°C but not at 0°C), transmembrane form of PrP (^{Ctm}PrP), a form not detected by Western blotting for PrP^{Sc} using the current standard methods (Hegde *et al*., 1999; Hope, 1999).

In some instances, one or more of these tests may be negative whereas others are positive thus leading to confusion as to the true diagnosis. Such cases may arise when the clinical signs are equivocal. They may also result following examination of tissues from clinically healthy animals. There is no consensus on the number of tests which should be positive in order to accept a diagnosis of a TSE/prion disease or TSE/prion infection (See Section III.l Introduction). There is no consensus on the action to be taken if a pre-clinical TSE/prion disease is demonstrated in a sheep or how the case should be reported. It is important therefore that agreement should be reached within the EU on the criteria for TSE diagnosis.

The following questions are relevant in the frame of what precedes:

1. Is there variation in the clinical signs of scrapie in sheep in the EU?
2. How much variation exists in the number and type of vacuoles detected in TSE affected sheep and which brain areas are affected?
3. Is the detection of PrP^{Sc} by IHC affected by the antibody used and by the details of the technique employed?
4. Similarly, is the detection of PrP^{Sc} by Western blotting affected by the antibody and the details of the methodology?
5. How sensitive and specific are these methods in comparison with each other?
6. How many of the tests (histopathology, IHC, Western blotting, SAF) need to be positive before a TSE diagnosis is accepted? Which test is essential within the minimum number acceptable?

A Shared Cost action at the EU level is working on these aspects as well as those in respect to clinical and preclinical diagnosis of TSE in sheep:

FAIR PL 98/7021 (Weavers, Ireland) aims to establish a European network for the surveillance of ruminant TSE and the standardisation and harmonisation of the process and criteria for the identification of suspected cases.

Discussion:

The clinical signs of scrapie can vary from obvious, typical signs to very subtle signs. In one study (Clark and Moar, 1992) 26 per cent of sheep found dead were diagnosed as scrapie positive post mortem. The authors concluded that scrapie cannot consistently be diagnosed on clinical signs alone even by experienced veterinary surgeons. The variation in clinical signs depends on a number of factors such as age, breed, PrP genotype, general health, stress situation, TSE strain or strains involved, duration of the clinical period of disease, etc. The clinical diagnosis of scrapie may be inaccurate or missed because of the diversity of clinical signs and the lack of agreement on the laboratory tests required to confirm a suspect clinical case leads to imprecision of diagnosis and lack of harmony on the scrapie status of sheep flocks in different countries. Clinical cases may be missed due to producers and veterinarians with preconceived ideas of what scrapie should look like (L.Detwiler, personal communication**)**. For example if the sheep does not scrape it can't be scrapie. That is wrong. For example Icelandic sheep with a form of scrapie called rida do not show pruritus. In recent years attempts have been made to describe the complex pattern of clinical features of scrapie. To this end, a number of video programmes have been made

by institutes in the EU, Norway and Iceland studying clinical scrapie. A video programme of the clinical signs of scrapie in sheep and goats using material from several European countries has been produced by the Scientific Veterinary Committee of the EC *via* MAFF UK with funding by the EC (MAFF 1998). The fair project PL-7021 is collecting and collating as many of these video programmes as are available. In a former SSC opinion it was advised that "didactic material of clinical signs of experimental BSE in sheep should be made, for example using the new series of experiments in sheep challenged with the BSE agent at the Neuropathogenesis Unit of the Institute for Animal Health, Edinburgh, UK. This study is now underway in collaboration with VLA, UK. Veterinary neurologists should be trained to study clinical signs of experimental BSE in sheep and to record the salient features on video-tape. A number of descriptive publications have been made in the recent years.

(Detwiler LA et al, 1996, Detwiler LA, 1992, Kimberlin RH, 1981, Toumazos P., 1991, Dickinson AG, 1976, Radostits OM et al, 1994, Kümper H., 1994**,** Wineland NE et al., 1998, Ulvund MJ, 1997, Ulvund MJ, 1999, Clark AM and Moar JAE, 1992, Onodera T and Hayashi T., 1994).

The oldest descriptions, which describe accurately the clinical signs of scrapie, and recognise its occurrence as a contagious disease in Britain in 1730 and in Germany in 1759, are still valid today. However, knowledge about scrapie remains insufficient and the real distribution and incidence of the disease is largely unknown, mainly due to difficulties in recognising the clinical signs and the inadequacy of the epidemiosurveillance networks. In the frame of the EU FAIR project PL 98/7021 a network has been set up. Task 4 of this project aims, especially in respect to sheep, to standardise the criteria for the clinical definition of suspect scrapie. The objectives of the project in respect to sheep TSE are to

- define the clinical signs associated with a "scrapie suspect case";
- build a practical decision tree for scrapie to help field veterinarians differentiate scrapie from other diseases that can be identified in the live animal;
- design databases to enable countries to monitor the variability in clinical presentations of scrapie and compare their findings;
- promote updates of available information and ways to disseminate information that is practical to users (farmers, veterinarians and animal health officers).

To give a general frame of all possible clinical signs, the definition of a "sheep TSE suspected" case should be as broad as possible. All "found dead" sheep over 1 year of age should be regarded as suspect in scrapie endemic areas.

No validated comparative test of laboratory diagnostic methods, has been carried out on sheep. There is however more information on the diagnostic tests used for the confirmation of BSE in cattle (Wells *et al*, 1998). It cannot be assumed that similar results would be found using these tests to diagnose sheep scrapie. However, the following can be stated:

SAF examination misses about 20-25% of the scrapie cases that are confirmed by histopathology, IHC or immunoblotting (M. Simmons, personal communication). However, SAF detection is still feasible on samples from scrapie-positive animals showing advanced autolysis (e.g.. fallen stock). Similarly, diagnostic investigations on autolysed material are possible by immunoblot detection of PrP^{Sc}.

In comparison to histopathology, immunohistochemistry (IHC) has the advantage that even in cases where microscopic examination of the brain is no longer reliable due to autolysis. This test is still possible even where relatively advanced autolysis has occurred. Possible problems with IHC concern a too high background staining and the properties of the antibodies used**.** In that respect a recent paper of Hardt *et al* (2000) immunohistochemical antigen unmasking and visualisation systems were compared and a variety of polyclonal and monoclonal antibodies to other epithopes on ruminant PrP were assessed, underlining the importance of the different steps and antibodies used.

Western blot analysis has a number of advantages, including possible application on autolysed material. It also permits glycopattern analysis to determine molecular strain variation. Similarly, IHC allows very early detection of PrP^{Sc} in sheep and it has the additional advantage of assessing the presence of PrP^{Sc} at cellular levels. However, precise results about the comparison of both the best versions of IHC and Western Blot are not available at present. Such comparisons are currently rather difficult to make as neither the IHC nor the western blotting technique is performed in European diagnostic laboratories using standardised conditions. Moreover, both methods have been improved recently by the introduction of novel visualization systems, the latest versions of which have not been compared. Furthermore, a variety of different protocols are currently used for the detection of PrP^{Sc} by Western blotting. These include or exclude SAF purification steps. The impact of these procedures on the PrP^{Sc} yields is currently unknown, but will be determined in the course of the EU funded project FAIR CT99 7021. Moreover, a UK study is underway to compare different methods for the confirmation of a clinical diagnosis of scrapie (Western blot, histopathology, IHC and SAF).

The detection of PrP^{Sc} in the peripheral lymphoid tissues e.g. tonsils (e.g., Schreuder et al, 1998, Roels et al, 1999) or third eyelid of incubating or pre-clinical animals using either IHC or Western Blot are not yet fully validated. There is some doubt regarding the use of the third eyelid as there appears to be reduction in the number of lymphoid follicles in older animals**.** The advantage of the third eyelid is that it is more accessible than tonsil and does not require a general anaesthetic for collection. Studies in the Netherlands have so far indicated that most, if not all, clinically healthy (including young) animals with IHC positive tonsils eventually develop disease. In the preliminary opinion on *Oral exposure of humans to the BSE agent: infective dose and species barrier* adopted by the Scientific Steering Committee on 2-3 March 2000, the link between PrP^{Sc} presence and infectivity, has been addressed.

Annex 1 provides a non-exhaustive matrix of methods applied for the confirmation of clinical and preclinical TSE diagnosis in sheep. (Bradley, personal communication, 2000). However, this chart should not be seen as limitative recommendation for the diagnosis of scrapie, especially in the framework of an eradication programme. For example (L.Detwiler, pers.comm, 2000) in the US, by policy and regulation, 4 lesions are required to be found before confirming scrapie. However if these lesions were identified in the brain, the sheep was positive regardless if anyone had observed clinical signs. Found dead may merely mean that the animal was not being observed closely. In most instances the history of the flock including animal movements supported the diagnosis of scrapie. The positives were never challenged. In 1992-93 a start was made with using IHC on brain tissue as a routine diagnostic tool in the US scrapie control program. An IHC brain positive is deemed a scrapied animal. Again, over the last eight

years there has not been a field case of an animal found to be IHC brain positive where someone has questioned the diagnosis, or the flock history has been such that the diagnosis would be questioned. If what was officially diagnosed as scrapie would have been limited to the recommendations suggested in the chart, a number of infected flocks would have missed.

Conclusions:

Microscopic examination of the brain does not guarantee the confirmation of diagnosis in all clinically suspect scrapie, especially if the material is autolysed and/or the normal presence of a limited number of vacuoles gives rise to some inconclusive results. The SAF method, compared to microscopic examination of the brain misses about 20-25% of the cases and is therefore not recommended unless on advanced autolysed material where SAF (Stack *et al*, 1993), together with Western blot analysis, are the only applicable tests.

Little up to date information is currently available on the comparative sensitivities of IHC and Western blot (WB) as both techniques have been substantially modified and improved recently. IHC and WB both have their uses, but not enough work has been done on absolute comparison in all circumstances and experts disagree. The Working Group recorded these disagreements, and noted that there are technical reasons for differences between laboratories. IHC has the advantage that tissue sections are readily available and may therefore be used as a first technique to re-examine pathologically suspicious samples. A detailed discussion on the particular sensitivities of PrP^{Sc} detection techniques should become possible when results from comparative studies become available.

In respect to the previous SSC reports and opinions on TSEs in sheep (cf "Context of the questions"), the Working Group concludes that no change can be proposed in the light of the present report.

Recommendation:

The Working group recommends that the continuation and extension of the above mentioned EU networks should be funded so that they can be continued beyond the current finish dates. To cover at maximum all possible clinical and pre-clinical TSE cases in sheep the following cases i.e. for eradication purposes, should be included: all clinical suspect cases, all clinical suspect cases or found dead animals above 1 year (FD) showing spongiform encephalopathy with classical histopathology (HP) alone, all clinical suspect cases of prion diseases or FD positive with immunohistochemistry or Western blot/Dot Blot (WB/DB) alone and all cases of pre-clinical TSE or FD scoring positive with histopathology plus one positive IHC or WB/DB test. SAF results should always be confirmed by an additional test (IHC, HP or WB/DB).

Remark: Referring to the draft non exhaustive matrix for the diagnosis of scrapie in sheep (annex 1), this chart should not be seen as limitative recommendation for the diagnosis of scrapie in the framework of an eradication programme. Eradicating TSE in a location or a country will would imply eradicating infection.

III.3. Discussion on the publication of T. Baron *et al* (1999).

Dr Thierry Baron presented his studies on PrP^{Sc} analysis in French scrapie cases. From 21 flocks which had outbreaks of scrapie, 42 scrapie sheep were tested and compared with 4 BSE affected cattle samples and 2 FSE affected cheetah samples. In addition, laboratory mice challenged and affected by BSE (cattle) and FSE (cheetah) were compared with C57BL/6 mice (from IFFA Credo) challenged and affected by C506M3 (mouse passaged) scrapie.

For western blotting and glycoform studies, several antibodies were used: the polyclonal anti-PrP peptides RS1 (against ovine PrP amino acids 98-113) and RB1 (against bovine PrP amino acids 105-120) and the monoclonal 3F4 (epitope hamster PrP amino acids 109-112).

Western blot analysis produces three bands in the final gel picture. These represent the unglycosylated form of PrP^{Sc} towards the foot of the gel, above that are the two mono-glycosylated bands, usually running so close together that they seem like a single wide band. Towards the top of the gel is the di-glycosylated form of the protein. No variation in fragment sizes on western blots was seen in the Baron et al (1999) scrapie cases although in C506M3 affected mice, the size of the unglycosylated PrP band was clearly higher than in BSE or FSE infected mice. There was therefore a concern that the French sheep affected by scrapie might actually be infected with BSE. Baron *et al* (2000) examined a Lacaune ARQ/ARQ sheep experimentally infected with BSE. On western blot analysis, the PrP^{Sc} from this latter sheep is different in appearance from that seen in PrP^{Sc} from natural sheep scrapie cases, but is more similar to that seen with BSE affected cattle. The work is continuing using SSBP/1 and CH1641 scrapie strains from the Institute for Animal Health, UK. CH1641 shows a similar glycoform profile to the BSE agent but is clearly distinguished from it by biological strain typing in mice. CH1641 is transmitted with great difficulty to mice whereas BSE agent from cattle transmits easily with a distinctive phenotype (Hope *et al*, 1999). It was agreed that there was a concern over delays with TSE work requiring animals and this resulted from the need for specialised animal accommodation.

Studies in Irish sheep with scrapie (Sweeney *et al*, 2000) and in Germany revealed similar findings in regard to glycoform signatures to those reported by Baron *et al* (1999).

The conclusion from the discussion was that there was therefore no conclusive evidence that French sheep were infected with BSE. It was agreed that the currently most accepted method for demonstrating the strain of TSE in the sheep was by mouse bioassay using the 4 mouse lines used at the Institute for Animal Health, UK. This work is presently underway (Baron, personal communication).

Points raised in the discussion concerned the following:

1. Genotypes of the French scrapie sheep: Not all animals were genotyped but those that were, encoded the VRQ and ARQ alleles of the sheep *PrP* gene as expected.
2. Technical points were raised concerning width of PrP bands on the western blots, the gap sizes between bands. Those with experience of the technique emphasised that despite careful attempts to keep the amounts of protein the same for each sample, this was in practice impossible. Different loadings of protein would produce minor differences in band appearance and a single blot was not enough to give a definitive result. Samples must be run next to each other on the same gel. In

addition, the visualisation step -the final step in the process which produces the black banding image on film - is also variable and crucial to control.

Conclusions:

1. It cannot be concluded that the study (Baron *et al*, 1999) demonstrates BSE in French sheep. It is therefore not necessary to revise the report of the working group on *The risk of infection of sheep and goats with BSE agent.*
2. Glycoform analysis on its own is not sufficient for strain typing in sheep.

Recommendations

It is recommended that similar work is undertaken in other EU countries to obtain more information on PrP glycoform profiles in sheep and goats with scrapie throughout the EU. It is also recommended that such studies should include non-EU European countries such as Cyprus, Iceland, Norway and Switzerland. It was noted that scrapie in Iceland was close to eradication.

IV. GENERAL CONCLUSIONS AND RECOMMENDATIONS

1. It is regarded as extremely important to encourage reporting of all scrapie cases.
2. As much relevant information as possible should be collected about each scrapie sheep.
3. It is noted that at present:
   - A clinical diagnosis of scrapie can only be made by clinical observation of live sheep by trained individuals familiar with the variation in the clinical presentation of the disease.
   - A pathological diagnosis of spongiform encephalopathy can only be made by microscopic examination of the brain.
   - A biochemical diagnosis of prion disease can only be made by positive detection of prion protein (PrP^{Sc}). However, the positive detection of PrP^{Sc} in tissues of a healthy sheep by a validated method may indicate that it is infected and likely to develop clinical scrapie if it lives long enough.
   - An aetiological diagnosis can only be made by determination of the biological strain type using a panel of in-bred strains of mice.
4. Table 1 in annex provides a draft non exhaustive matrix for the diagnosis and confirmation of clinical and preclinical TSE in sheep (Bradley, personal communication, 2000). No single test is sufficient on its own to confirm a diagnosis of scrapie/TSE in sheep. To cover at maximum all possible clinical and pre-clinical TSE cases in sheep the following cases i.e. for eradication purposes, should be included: all clinical suspect cases, all clinical suspect cases or found dead animals above 1 year (FD) showing spongiform encephalopathy with classical histopathology (HP) alone, all clinical suspect cases of prion diseases or FD positive with immunohistochemistry or Western blot/Dot Blot (WB/DB) alone and all cases of pre-clinical TSE or FD scoring positive with histopathology plus one positive IHC or WB/DB test. SAF results should always be confirmed by an additional test (IHC, HP or WB/DB).

Remark: Referring to the non exhaustive matrix for the diagnosis of scrapie in sheep (annex 1), this chart should not be seen as limitative recommendation for the diagnosis of scrapie in the framework of an eradication programme. Eradicating TSE in a location or a country would imply eradicating infection.

5. It cannot be concluded that the study of Baron *et al*, (1999) demonstrates BSE in French sheep. It is therefore not necessary to revise the report of the working group on *The risk of infection of sheep and goats with BSE agent* included in the opinion of the SSC of September 1998 on the same subject.

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ANNEX 1:

DRAFT NON EXHAUSTIVE MATRIX FOR THE DIAGNOSIS AND CONFIRMATION OF CLINICAL AND PRECLINICAL TSE DIAGNOSIS IN SHEEP USING CLINICAL FEATURES AND EXAMINATION OF THE CNS

(Bradley, personal communication, 2000)

DIAGNOSTIC METHOD/ DIAGNOSIS	CLINICAL	MICROSCOPIC EXAMINATION OF BRAIN	IHC	WESTERN BLOT/ DOT BLOT	SAF DETECTION
CLINICAL SUSPECT	+	ND	ND	ND	ND
SPONGIFORM ENCEPHALOPATHY (SE)	ND or FD	+	ND	ND	ND
PRION DISEASE/SE	ND or FD	ND	+	ND	ND
PRION DISEASE	ND or FD	ND	ND	+	ND
SUSPECT SCRAPIE OR SUSPECT PRION DISEASE	+ or ND	ND	ND	ND	+
SCRAPIE	+	+	ND	ND	+ or ND
SCRAPIE	+	+ or ND	+ (one or more PrP^{res} detection methods)		+ or ND
SCRAPIE	+	+	ND	ND	+
PRE-CLINICAL SCRAPIE	Clinically healthy or FD	+	+ (one or more of these detection methods)		

(ND = not done) **(FD = found dead above 1 year of age)**

Notes:

1. Since scrapie is a clinical condition there must be clinical neurological signs.
2. A diagnosis of scrapie requires positive clinical signs or found dead without previous clinical inspection and at least one other positive result other than SAF detection alone.
3. Another factor to consider could be transmissibility. A positive transmission might permit an etiological diagnosis or confirm a TSE if CNS lesions were present. It could only confirm scrapie if clinical signs were present. Otherwise it would be 'scrapie infection', ''prion infection' or 'prion disease', according to the other findings present.
4. Molecular analysis of strains has been avoided in this table, and is premature, as criteria to determine strains by this means have not yet been agreed.
5. In field investigations, positive SAF or PrP^{res} findings alone and only outside the CNS can lead to an indication of prion infection.
6. All methods require criteria for interpretation of a positive result**.**

Remark: this chart should not be seen as limitative recommendations for the diagnosis of scrapie, especially in the framework of an eradication programme. (See text).