EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C1 - Follow-up and dissemination of scientific opinions

STRATEGY TO INVESTIGATE

THE POSSIBLE PRESENCE OF BSE IN SHEEP

ADOPTED BY THE SCIENTIFIC STEERING COMMITTEE AT ITS MEETING OF 4-5 APRIL 2002

OPINION

BACKGROUND AND MANDATE:

In its opinion of 19 October 2001 on *The safety of small ruminant products should BSE in small ruminants become probable / confirmed*, the Scientific Steering Committee (SSC) signalled that risk assessments related to the possible presence of BSE in small ruminants was hampered by a lack of basic information on the incidence of TSEs in small ruminants and by the statistically very limited number of straintyping results of TSEs in small ruminants. In its opinion of 30 November 2001, the SSC listed the requirements for statistically authoritative BSE/TSE surveys. These are currently being implemented and are expected to result in a better knowledge of the TSE incidence in small ruminants in the European Union.

As a further follow-up to the SSC opinion of 19 October 2001, Commission Services invited the SSC to suggest a strategy to investigate, by mouse strain typing or any other suitable method, whether BSE is present in the small ruminant population in the Community. The strategy should include details on:

- Strain-typing to be used (if mouse strain typing is recommended, details on the protocol);
- Are there other techniques to be explored, e.g., molecular methods if existing or under development or having the potential to become rapidly operational.
- Methods for sample selection and number of samples to be analysed per Member State

Other relevant information (for example, if each animal should be investigated individually or if pooled samples can be used).

The SSC asked the TSE/BSE *ad hoc* Group to prepare, in the light of current knowledge, a detailed report addressing the issues related to a targeted investigation for the presence of BSE in sheep, that would serve as a basis for an opinion on the above questions.

The report is attached and the SSC concludes as follows:

CURRENT METHODS:

Currently all methods of typing or discriminating between agents of the TSE rely upon phenotypic characteristics of the disease caused, either on transmission to laboratory animals, or in the host species.

Biological strain typing has been developed principally by transmission of scrapie from sheep sources to inbred mouse strains, using incubation period and brain pathology as criteria. It has been used principally as a tool for research into the nature of the scrapie agent. More recently the approach has been applied to generate data on field strains of TSE and to establish links between natural TSE's in different species.

Strain typing has been applied specifically to characterise of a TSE agent from a natural host source. Uniquely in the use of the method, BSE isolates from cattle, and after a single pass in certain other mammalian species, result in a constant phenotype of disease on primary transmission to a panel of mice. If required, confirmatory evidence of the BSE agent "signature" is given by a single subpassage in VM mice to obtain the 301V mouse adapted/selected agent strain.

There is a high degree of confidence that this approach, accompanied by appropriate statistical analysis, would identify the BSE agent from sheep clinically affected with BSE after infection from a cattle source. Failure to match ensures neither the absence of the

BSE agent in the isolate nor that the agent characterised is not derived from the BSE agent during a previous passage. There is, however, uncertainty whether the approach would be successful in identifying the BSE agent if it had been sequentially passaged naturally (like scrapie) in small ruminant species. This problem is confounded by a lack of information on the number and diversity of strains of scrapie agent in small ruminant populations. Furthermore, this approach:

- Has the potential to be compromised by preclinical material; requires high titre brainstem sample preferably from clinically affected cases, taken shortly after death avoiding any risk of contamination with other TSE agents or bacteria.
- It is time consuming (<2years)
- It is costly, requires investment in rigorous training/standardisation for successful transfer between laboratories.

Applying similar phenotype characterisation to transmissions of TSE sources in transgenic mouse models is under development.

Molecular methods of typing TSE sources are based upon phenotypic properties of the host's disease specific protein during biochemical denaturation. Several such methods, applicable to brain material, have the potential to differentiate BSE and scrapie sources, but require validation or further development. These approaches:

- Require a maximum (for multiple test/repeated testing) of 10-15g of brain tissue.
- Allow testing of large numbers of source cases on a time scale of days.
- Were developed for use in clinical cases. Currently, development extending to lymphoid tissue and preclinical applications.

Characterisation of host pathology, particularly those features defining phenotype on the basis of PrP immunohistochemistry, offers prospects for differentiating BSE and scrapie sources. These would require fixed tissue, probably whole brain and samples of lymphoid tissues.

SUGGESTED STRATEGY:

In spite of the limitations presented above, the SSC suggests the following strategy for the investigation of the presence of BSE in the Community sheep population is to proceed in 3 stages:

Stage 1:

a) Possible use of evaluated rapid tests on brain sample of statistically representative slaughtered populations of healthy small ruminants, to establish occurrence/prevalence of TSE in sheep +/- geographical targeting based on BSE risk.

b) Possible application of such testing to fallen stock/casualty slaughtered animals, to supplement a) and provide material from clinical cases.

c) Possible identification of suspect clinical cases from notification of cases of scrapie (passive surveillance, with incentive/compensation) to also provide clinical case material.

Stage 2:

Application of at least two molecular typing methods to material from all positive cases resulting from 1. Given the relatively low incidence (in statistical terms) of TSEs, it is recommended that all animals found positive in the first stage, are also submitted to the second stage testing.

Note: the outcome of a single molecular typing approach will not provide proof beyond any doubt that BSE is present or excluded. It is therefore recommended to use at least 2 molecular methods to verify whether or not the positive TSE case found in stage 1 is BSE-like. Given the current state of development of these molecular methods, it is also not yet possible to issue strong guidelines on which methods to use or which discriminatory thresholds to apply. Therefore, should the results obtained in a given laboratory provide indications that the TSE agent has a BSE-like characteristic, these should be submitted for independent confirmation to an expert panel and/or to another laboratory. If this panel or laboratory confirms the finding, and pending the outcome of mouse transmissions in Stage 3 (see next), the presence of BSE in small ruminants should be considered as being probable and the geographical extent of the risk would need to be assessed (i.e., EU-wide or confined to a country, region, flock or single animal). The Scientific Steering Committee is preparing an opinion on the latter.

Stage 3:

Application of mouse strain typing for the identification of BSE-like isolates from the results of 2. If results from stage 2 indicate unsatisfactory samples for mouse strain typing, consideration should be given to ways of obtaining additional material for strain typing as at 1b) and 1c). In conjunction with identification of a case of TSE in small ruminants, with BSE-like characteristics, on molecular typing, further investigation of the flock / geographic region of origin of the case should be conducted. This should include additional testing of clinical cases and epidemiological investigation (history of TSE occurrence, feeding practices etc.). Mouse strain typing specifically for the identification of BSE-like strains should be applied only to single cases of TSE in small ruminants; results from pooled samples may prove uninterpretable.

OTHER ISSUES:

The methodological details for biological strain typing and other relevant information (for example, whether each animal should be investigated individually or if pooled samples can be used) are provided in the attached report.

It is unlikely that there exists within every country a laboratory that would be sufficiently equipped and with the appropriate experience to perform the second and third stage investigations. A well structured laboratory system or network will therefore need to be established, for example as proposed in the attached report.

The outcome of a single molecular typing approach will not provide proof beyond any doubt that BSE is present or excluded. Given their current state of development[1] it is also not yet possible to issue strong guidelines on which methods to use or which discriminatory thresholds to apply. Should the results obtained in a given laboratory provide indications that the TSE agent has a BSE-like characteristic, then they should be

[1] Which implies also that comparability of results between laboratories has not been verified and that appropriate availability of standardised reagents and antibodies is not necessarily guaranteed.

peer-reviewed by an appropriate expert panel. Similarly, subsequent biological strain typing results should be reviewed by such a panel.

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THE POSSIBLE PRESENCE OF BSE IN SHEEP

REPORT OF THE TSE/BSE AD HOC GROUP

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I. MANDATE

As a follow-up to its opinion of 18-19 October 2001 on *The safety of small ruminant products should BSE in small ruminants become probable / confirmed*, the SSC was invited to suggest a strategy to investigate, by mouse strain typing or any other suitable method, whether BSE is present in the small ruminant population in the Community. The strategy should include details on:

- Strain-typing to be used (if mouse strain typing is recommended, details on the protocol);
- Are there other techniques to be explored, e.g., molecular methods if existing or under development or having the potential to become rapidly operational.
- Methods for sample selection and number of samples to be analysed per Member State
- Other relevant information (for example, if each animal should be investigated individually or if pooled samples can be used).

II. REPORT

II.1. PREAMBLE

1. The various characteristics which make up our incomplete understanding of the nature of the transmissible spongiform encephalopathy (TSE) agents prevent typing of individual isolates of such agents by conventional microbiological methods. Notably methods based on immunological or genomic factors cannot be applied. Evidence, nevertheless, that multiple strains of TSE agents exist has come from transmission and serial passage of sheep scrapie agents largely in inbred mouse lines. Similar evidence has come from passage of transmissible mink encephalopathy (TME) in hamsters. For scrapie, agent strain discrimination has been based on disease characteristics, that is the phenotype of disease expressed in inbred mice. These characteristics have included incubation period and the patterns of distribution and relative severity of the spongiform changes in the brain (the lesion profile). Strain characterisation has been defined mostly in terms of the reproducibility and stability of the disease phenotype on serial transmission in a specified mouse genotype. Thus, an individual "strain" might be defined as an isolate which consistently matches certain agreed criteria or characteristics. Clearly, the failure of some sheep scrapie to transmit to mice excludes certain field strains of scrapie from characterisation by the method. It should be emphasised that this method was developed and used historically, principally in a research context to inform on the nature of the causative agent of the TSE's. More recently the approach has been applied to inform on field strains of TSE and to establish links between natural TSE's in different species.

   With the advent of BSE and its successful transmission to a panel of mouse lines came the finding that at each primary transmission a closely similar disease phenotype emerged. This together with other evidence from the constant pattern of the pathology of BSE in cattle led to the conclusion that the BSE epidemic was sustained by a single, stable, cattle adapted strain of a scrapie-like agent. Furthermore, the uniformity of the BSE phenotype in the panel of mouse genotypes was also maintained after a putative natural or experimental single pass of the BSE agent in several other species, including

the domestic cat, greater kudu, nyala and domestic pigs and sheep and latterly, man. The biotyping of BSE and vCJD agents has, therefore, in stark contrast to the corresponding work with scrapie of sheep, been made largely upon primary transmission from the host species and unchanged phenotypic characteristics of BSE isolates in mice after a single pass in a number of other mammalian species. This difference is important in the general concept and in the confidence with which such methods can be applied to the identification of BSE-like agents, isolated from other animal species. With regard to biological strain typing in mice there is sufficient evidence to suggest that an isolate can be confirmed to be BSE-like if a complete match is obtained with an agreed full set of characteristics which have been defined repeatedly for the BSE agent. Dependent on the degree of matching, when the characterisation features share some of the essential elements, an isolate might be subjected to further investigation. Failure to match does not ensure the absence of the BSE agent in the isolate nor that the agent characterised was not derived from the BSE agent during a previous passage.

This biological strain typing approach has been the only way of attempting the discrimination of TSE strains, but more recently molecular methods have been applied to characterise the properties of the disease specific form of the protein PrP (which may represent the major or sole molecule of the infectious agent)in the host. It must be accepted, however, that while uncertainty regarding the nature of the agent remains, such methods, like those of biological strain typing, provide only a further dimension to the pathological phenotype of the disease. The variation in expression of phenotypic characteristics may be large. This is particularly so in the case of sheep scrapie isolates. There is overlap between the ranges of values of molecular characteristics of different defined strains. Furthermore, the biological relationship between the biotyping and molecular phenotyping is not known. Comparisons between the results of these differing approaches have been made particularly in respect to the segregation of human forms of TSE and the similarities between vCJD and BSE isolates and one of the complexities of such comparisons is the questionable validity of making them, irrespective of, or across, the passage history of the isolate.

Finally, there is the possibility of defining “strain” through characterisation of the phenotype of disease in the host species. This has broadly defined the “origin” or “type” of some human prion diseases and among the animal TSE’s, it has been possible to characterise the pathology of BSE as being distinct from that of experimental scrapie transmissions to cattle.

At present, strains of TSE agents have to be defined operationally in terms of disease phenotype by mouse transmission. Although a molecular understanding of strain variation defined in this way is lacking, the method is currently the most discriminatory means and is therefore the “gold standard”. Clearly, this does not rule out the possibility in future of other methods that are more discriminatory, with consequent change in the “gold standard”.

A major epidemiological interest is in the zoonotic potential of a TSE source, but pathogenicity cannot be assessed from strain typing. Nevertheless, all available knowledge suggests that the BSE agent, defined by a full set of characteristics on transmission to inbred mice, is pathogenic for humans. In that it is, as yet, the only animal TSE source recognised with such properties, isolation of this strain of agent provides the only practical basis of identification of a source as a hazard to man. It is important here to stress that,

as indicated above, an isolate should be confirmed to contain a BSE-like strain only if a complete match is obtained with an agreed full range of defined strain typing criteria. Defining the boundaries of the criteria needs statistical input[2] which is currently being developed. For operational purposes an individual component of strain typing (e.g. incubation period characteristics in a single mouse strain, or elements of the lesion profile) may be said to have a BSE-like characteristic, without implication of the source being BSE. Similarly, until defined more precisely, any molecular typing characteristic should be ascribed the latter terminology.

Note: With respect to the conclusions and concerns of the SSC report of 14 April 2000[3] that « *comparisons of the signatures of PrP^{Sc} should be interpreted with great caution and (…) that glycoform analysis on its own is not sufficient* », the WG considered that they remain valid. Concerning the statement in the same report that « *differential biochemical strain typing is not yet possible and results should be interpreted with caution* », the WG considered that differential molecular typing of sources of TSE may be a useful approach but is not, on its own, sufficient.

2. Critical to the ability to identify the BSE agent in small ruminant TSE isolates are the principles of strain stability on passage in the same and different hosts. In rodent models (see Bruce 1996, for review) for example: 1) mouse adapted scrapie and BSE agents generally remain stable on passage at high dose in the same mouse genotype; 2) some well characterised mouse strains remain unchanged on passage in a different mouse genotype, while others undergo change slowly over serial passage and 3) some strains also remain stable on passage through another species, while others undergo change.

   It is generally accepted that, should the BSE agent be present in small ruminants, it entered these populations via feed containing contaminated animal proteins. If it was subsequently propagated and amplified, the mechanism of propagation was probably via vertical or horizontal routes[4]. Because of the feed-bans that have been progressively implemented during recent years, it is unlikely that primary infection via feed would currently be responsible for a significant part of the possible BSE infected population amongst the total TSE-infected small ruminant population in the UK. In mainland Europe given the later total ban on use of MBM, and possible greater numbers of milking sheep which would receive supplementary feed, the likelihood of BSE infection being primary is relatively higher.

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[2] A database needs to be developed which informs about how many of the 'admissible' test samples overlaps with BSE-like strain on criterion A [or B or C] how many on pairs of criteria A&B [or A&C] . . . or what is the distribution of discriminant score X [based on criteria A to Z] for admissible TSE samples which are NOT BSE-like in strain, etc. Thus, statistical input/discrimination will be evolutionary.

[3] Report of the Scientific Steering Committee of 13-14 April 2000 on *The criteria for diagnosis of clinical and pre-clinical TSE disease in sheep and for differential biochemical diagnosis of TSE agent strains*.

[4] See the SSC opinion of 29-30 November 2001 on BSE transmission.

This means that the BSE agent, if (still) present, could be[5] the result of one or several "sub-passages", resulting from possible horizontal/maternal transmission, in subsequent generations of animals after an initial infection via feed several years ago.

The BSE agent strain has been characterised from observations of known BSE from cows or after one passage in certain genotypes of sheep/other species. These isolates were strain typed from small numbers of animals in the early part of the epidemic in the UK (and Switzerland) so there is little direct evidence of BSE stability on repeated passage through a single species. However, there is the assumption that the recycling of the BSE agent through feed will have resulted in several passages in cattle in the course of the epidemic, with no clear indication of any substantial change in disease phenotype.

Very limited documentation is available on whether the BSE agent characteristics remain unchanged during passages in sheep: Houston et al (2000) suggest that BSE can be diagnosed at the second passage in sheep (a sheep which developed a TSE after transfusion with blood from a BSE infected sheep), but this is based only on the evidence of the brain material of the sheep having a glycoform pattern on WB comparable with that obtained with BSE strain sources. No mouse strain typing information is yet available from BSE passaged more than once through sheep. Also, stability of BSE on serial passage in sheep may differ according to the genotype of the sheep – we do not yet have direct experimental evidence on this. The number of experimental cases of BSE infected sheep or goats, which have been transmitted and characterised using transmission in mice is very low. Two cases showed a typical BSE lesion profile (Foster et al, 1996). Furthermore, a limited number of field scrapie isolates which did not show a BSE profile have been fully described in the literature (Bruce et al, 1994; Bruce, 1996; Bruce et al, 1997; Fraser et al, 1992; Bruce et al. 2002).

The evidence that on a single passage in some other species, the phenotype of disease in mice does not change, might favour the argument that the BSE agent could remain stable on transmission in the majority of new host species.

TheWorking Group considers that data on biotyping after primary infection of sheep with the BSE agent are sufficient to form the basis of a strategy to identify the BSE agent in sheep under field conditions, should it be present. Change of the agent strain on passage in small ruminants is a possibility, which at present would not be resolvable by the application of such an approach. Because of insufficient information about the diversity of extant natural scrapie agents there is also the theoretical possibility of pre-existence of the BSE strain in sheep populations. For example it has been suggested that

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[5] It is not excluded that (cf BARB cases in UK and BSE cases in Mainland Europe born in 1997) sheep could still present clinically with BSE after being primarily infected following exposure to BSE contaminated MBM, or, theoretically, that environmental exposure could occur from soils contaminated with the BSE agent. Therefore, if BSE occurs in sheep, it is not excluded that such cases would be a mixture of primary infections and sub-passages of the BSE agent. If this were the case and after passages the agent is not 100% stable, then the identification of these strains or isolates as being really BSE derived would represent a major problem.

"*BSE prions in sheep may (…) have been there all the time at very low levels that pose no significant risk to humans but unusual circumstances might have allowed them to spread either through the sheep or cattle population and accumulate to levels hazardous to humans."* [6] Irrespective of the theoretical source of the agent the recognition of a BSE-like strain in small ruminants would inevitably evoke the same practical outcome.

II.2. METHODS FOR TYPING TSE STRAINS IN CATTLE AND SMALL RUMINANTS.

Preamble:

From the limited number of BSE samples (from Great Britain and Switzerland) transmitted and strain typed in mice it is concluded that each contained the same major strain. This does not inform on the epidemic as a whole, because of the few samples, but the fact that BSE in cattle is a relatively uniform disease would suggest that there is little strain variation in the causative agent. It should be emphasized also that none of the molecular methods discussed in the next sections have been used for typing of material from a large series of cattle. Only a limited series of cattle samples have been examined as BSE controls to compare with other species.

Based on the results of biological strain typing in mice, the BSE strain properties are considered unique and this is the basis of any approach to identify the BSE strain. At the present stage of knowledge it remains problematic to attempt, on anything but a research scale, full characterisation of isolates from any species where the mouse strain typing characteristics do not fulfil agreed criteria for the BSE strain on primary transmission to mice. Devoting resources to the strategy of identifying a clear BSE signature in the primary transmissions is the only realistic course of action, at least until we have some information concerning the biological characteristics of serially sheep-passaged BSE. It is possible, for example, that there may be some sources which contain a mixture of scrapie and BSE strains and these would be expected to transmit to mice in a complex manner. Exploring how this is reflected in the mouse-to-mouse subpassage data is appropriate only in a research context.

It must also be emphasised that both molecular typing and biological strain typing have been established on examination of relatively high titre brain material from clinical source cases. There are reservations in particular, about attempting to strain type on preclinical (apparently healthy animals) material collected in the course of prevalence surveys, because it may be difficult to interpret transmissions from samples with potentially very low and variable titres. In this situation, it could be anticipated that there would be a high proportion of negative transmissions and, in the positive transmissions, incomplete lesion profile data in the shorter incubation period mouse strains. Given the potential consequences of a positive identification this would not be enough evidence on which to confirm BSE in small ruminants. However, recognising the problems inherent in acquiring suspect clinical cases, it may be necessary to review the strategy for detection of BSE in small ruminants, dependent upon interim results utilising material from slaughter surveys. Nonetheless, a survey based on random sampled

[6] Hypothetical scenario suggested by Stanley Prusiner's in his letter of 2 August 2000 to the SSC secretariat.

clinical scrapie cases for mouse transmission, which had first been screened with whatever laboratory diagnostic method deemed to be appropriate, would be a preferable approach, and may ultimately prove essential to achieving the objective.

II.2.1. Biological strain-typing by transmission in animal models.

Investigation of the biological properties of TSE isolates is currently the only available and well documented method of strain typing (Bruce 1996). These biological properties include the incubation periods and patterns of neurological targeting (the "lesion profiles") in brain, which differ according to the TSE strain and the animal line (particularly PrP genotype) into which the source was introduced, providing other variables, such as route of exposure, are kept constant. In practice the route(s) of exposure used are i/c, or combined i/c and i/p injection.

For identifying scrapie strains and BSE, groups of various lines of inbred mice have been used. Examples of the mouse lines used are RIII, C57BL, VM and VMxC57BL. Such a group of mouse lines is called a "panel". Stable differences between incubation periods and lesion profiles observed after passage of an isolate in the various lines has provided information permitting the definition of a number of mouse adapted strains of scrapie which may inform on the strain of agent in the sheep host. To obtain a stable, clear and unambiguous set of characteristics [incubation periods and lesion profiles] it is necessary to conduct primary and then a series of subsequent inoculations ("passages"). After firstly infecting a panel of mice with material from the original host animal under investigation a second panel of mice is inoculated with material from the first panel. The strategy for this sub-passage and, if necessary, subsequent biological cloning of strains is discussed elsewhere (Bruce, 1996). On second or subsequent passages the characteristics of incubation periods and lesion profiles may change, with "adaptation" of the agent to the new species/genotype. Typically in the course of such passages the characteristics become stable with most, if not all, animals developing the disease within short and consistent incubation periods with a constant mean lesion profile. Only stability of the strain properties on passage and reproducibility of the entire characterisation process will allow conclusions to be drawn with regard to the strain present in the source animal. Propagation in a given mouse genotype exerts a selection pressure that may not permit the original wild type agent to be selected. Nevertheless, previous work on strain stability across different host species/genotypes would suggest that, if a different strain is isolated in mice than the one(s) present in sheep, some molecular relationship between the informational molecules of the agents in the two species would be expected. The concept here is that isolates never contain a single, molecularly distinct strain – that, like viruses, a strain will give rise to variants and so an isolate will consist of a population of "quasi-species". Transmission to mice may favour a minor variant, but it is still likely to be related to the predominant strain in the sheep, unless the sheep has been infected from more than one unrelated sources.

Uniquely, in the history of the use of the method, the consistency of the biological characteristics of BSE in mice on primary isolation, or after a single pass in certain other species, has enabled identification of the BSE agent in an isolate by a single transmission to mice. This recognisable and repeatable BSE pattern has not been seen in a range of transmissions of (presumably) unrelated

TSEs from other species. And the other species (cat, kudu, nyala, human), from which transmissions have produced the same pattern, have epidemiological links with BSE. For the purpose therefore, of identifying the BSE strain, a primary passage and three mouse lines have been proposed in the simplified protocol described in the Annex[7]. This provides a summary of a simplified protocol for biological strain-typing of scrapie and BSE developed by the Neuropathogenesis Unit, Institute for Animal Health, Edinburgh. Such a simplification results from the observation that, in most cases, a BSE source transmits to all inoculated mice at the first passage. This is contrary to experience with most scrapie sources. The relationship between murine scrapie strains and sheep scrapie strains is still uncertain. Although several murine strains have been derived from natural sheep scrapie sources it is currently unclear just how these murine strains relate to sheep scrapie strains and just how many natural field sheep scrapie strains may exist.

Two experimental sheep scrapie isolates have previously been described: SSBP/1 and CH1641 are sheep passaged isolates characterised by workers at NPU. The passage history of these isolates prevents any conclusions as to their similarity to natural disease. Part of the problem in the identification of ovine field TSE strains which may resemble BSE is the lack of information about the number and diversity of sheep scrapie strains although this may not be as great as once it was considered. Not all scrapie strain types are readily transmitted to mice. In a recently published study (Bruce et al. 2002), albeit from a small number (10) of natural scrapie cases**,** only 2 mouse-passaged strains were isolated.

Several practical aspects of conducting the strain typing protocol must be considered:

a) Care should be taken in ensuring sufficient numbers of mice in each transmission group so as to allow appropriate statistically significant analysis of the results. The minimum number of test animals needed will depend upon the variability of the characteristics investigated and should permit calculation of a mean value that is statistically different from that obtained for another strain. In practice a minimum of 20 mice per group is advised.

b) To define this more accurately, information from enough sources/ isolates/strains has yet to be obtained and collated. A standard control panel of TSE sources/isolates/strains against which the method can be evaluated is required.

c) Further controls for the method, such as a standard set of mouse brain sections for a given lesion profile, though not as yet available as reference material could, more easily be produced.

d) Reference material in the form of standard lesion profile graphics with appropriate application of multivariate statistical analysis to define agreed limits of the profile are also needed.

e) In addition to the biological variation that may be experienced in lesion profiles, mention needs to be made also of the potential within the method of technical and observer variation. Because the quantitative assessment of the neuropathology in mouse brains is subjective, reproducibility is

[7] [to be verified with NPU] The protocol requires to be supplemented with SOP's supplied by NPU.

dependent on careful definition of lesion scoring and is achieved most readily with a single observer throughout a study. The obvious problems of training and standardisation make the method labour intensive to transfer between laboratories.

f) As the outcome of biological strain typing depends upon measuring a long incubation period and on multiple sub-passages of isolates, it is a very time-consuming and costly process. If strain typing of BSE were based on primary transmission results in RIII, C57BL and VM mice, the time-scale is usually slightly less than 2 years. Basing the initial assessment on the incubation period and lesion profile in RIII mice alone could be shorter than 1 year for the transmission component. It then relies on the efficiency of the procedures thereafter as to release of results. If sub-passage in VM mice is required, then the incubation period of BSE in VM mice on primary transmission followed by a 3 month incubation period in VM mice on second passage will mean 18 months minimum to identify the strain 301V, assuming secondary passage is started immediately from the initial VM mice to succumb to primary infection.

There are, as yet no reports of the features of the phenotype of disease in an appropriate panel of mice following serial passages of BSE in sheep or goats. As the outcome of such experiments may well differ between PrP genotypes (or permutations thereof) of donor sheep, no single study will provide definitive answers.

There are no published data on the possible discriminatory power of immunohistochemical patterns of PrPres in the brains of mice as an adjunct to the lesion profile based on vacuolar pathology. Bruce et al., (1989) have shown precise targeting of PrP accumulation in mouse scrapie, similar to the targeting of vacuolar pathology but little work has been published on the quantitation and profiling of PrP immunostaining in mouse models.

<u>Note on the possible use of transgenic mice for biological strain-typing</u>:

New methods for biological strain-typing based on the use of genetically engineered animals are currently under development. For TSEs in sheep for example, genetically modified mice ("Tg") are used in which the gene that produces (mouse) prions, is replaced by the corresponding gene, from sheep. In such test animals, the species barrier is assumed to be considerably lower, or even absent, and the incubation period is indeed significantly reduced compared to wild-type mice. In some experiments, the incubation time in mice after transmission of a sheep scrapie source was indeed as short as approx. 70 days (Vilotte *et al*, 2001), but this is not the general rule. Other studies showed mean incubation periods between 240 and 500 days, depending on the scrapie sources tested (Crozet et al., 2001a, 2001b). It is accepted that transmission of sheep TSE to transgenic mice, if successful, occurs within 1 year in most cases. The transmission of BSE from experimentally infected sheep was reported in a single experiment in an ovine (ARQ sequence) transgenic mouse line (Tg4), with a mean incubation period of 300 days (Crozet et al., 2001b). Interestingly, this transmission showed abundant “florid plaques” in the brain of transgenic mice with this sheep BSE isolate (genotype homozygous ARQ), whereas no such plaques were observed in any of the six different natural scrapie sources also transmitted to these transgenic mice. It is noteworthy that these lesions were

identical to those which allowed the recognition of the variant Creutzfeldt-Jakob disease in human. [Florid plaques have also been observed in wild type mice in 3/5 transmissions of Icelandic scrapie and in a transmission of UK scrapie from a single Suffolk/Colbred cross sheep (Fraser, H., 1983; McBride, P.A., Bruce, M.E., Fraser, H., (1988). Similar lesions were also observed following the BSE transmission into macaques (Lasmezas et al. 2001)].

However, only a single experimental BSE in sheep isolate has so far been transmitted to these mice. The sensitivity and specificity of the presence of these florid plaques in ovine transgenic has to be re-examined before it can possibly be considered as a reliable indicator for the finding of BSE in sheep. The behaviour of isolates such as CH1641, which shares some biochemical properties with BSE, is also still unknown in these transgenic mice. It is of interest that the lower molecular weight of proteinase K resistant PrP was also maintained in ovine transgenic mice following BSE transmission, in contrast with the six natural scrapie sources/isolates.

II.2.2. Molecular methods for TSE typing under development.

Molecular methods for typing TSE sources are based on the properties of disease-specific protease-resistant fragments of PrP (PrP^{Sc}) or physico-chemical behaviour of the fragments e.g. during denaturation. These methods allow analysis of the phenotypic characteristics of the PrP related to the host disease to be performed within days. Preliminary results of several of the methods are promising and compared to murine transmission studies, they can be applied on a much larger scale and can be performed more rapidly. However, none of the methods has undergone any formal validation and none has thus been converted into a routinely applicable test. Also, the tests listed hereafter have not been evaluated for their ability to distinguish between BSE and scrapie. Some of the work has only been done on experimental scrapie and a very limited number of isolates from experimentally BSE infected sheep, all at primary passage.

The various molecular approaches for the potential differentiation of scrapie and BSE sources can be classified into the following groups:

a) <u>Methods using the proteinase K cleavage site:</u>

As initially described in humans with variant Creutzfeldt-Jakob disease as well as in mice infected with BSE, a lower molecular mass of proteinase K resistant PrP has been found in BSE infected sheep. This has been shown to be associated with a different cleavage site by proteinase K during the extraction of resistant PrP (Parchi et al., 2000; Baron et al., 2001). In sheep this has been described in three separate Western blot studies from British (Hill et al., 1998; Stack et al., in press) and from French scrapie cases (Baron et al., 2000). The number of BSE infected sheep used in these studies was very limited.

An Elisa test, based on the same principle as the Biorad Elisa diagnostic test for cattle BSE, has also been developed, which compares the detection of PrPres using different proteinase K digestion conditions (J. Grassi and J.-P. Deslys, unpublished data). It is however already known that the lower molecular weight of PrP res (around 19 kDa) is not fully specific for BSE. Indeed, the CH 1641 experimental scrapie isolate shows a closely similar

PrPres pattern and particularly with regard to molecular mass, compared to BSE, whereas this isolate strongly differs from BSE by its transmission properties in mice. This same low molecular weight was also observed in ovine transgenic mice infected with brain tissue from BSE experimentally infected sheep (Crozet et al., 2001b), which would suggest that the biochemical properties could also be maintained during serial transmission in sheep, at least in the same genotype. So far, no natural scrapie isolate has been reported in the literature with a PrP^{res} pattern identical to that of BSE infected sheep.

M.Stack at the UK Veterinary Laboratory Agency (VLA) at Weybridge has been studying modifications of the Western Blot technique to try and distinguish between scrapie and BSE infection. Also here, preliminary results are promising (Stack et al, in press). This approach uses the principles of molecular weight estimation and glycoform ratio (Collinge et al.,1996) in combination with differential staining of the bands with two monoclonal antibodies. Using the Prionics-Check test, with a centrifugation step to remove larger particles and employing monoclonal antibodies 6H4 (Prionics) or P4 (Groschup, Tübingen), results suggest that while scrapie sources and sources of BSE from bovines or ovines stain with 6H4, sources of BSE from bovines and ovines do not stain with P4. This is in contrast with results with P4 in other test formats. This finding conforms with the interpretation of data from Grassi and Deslys that BSE and scrapie in sheep can be distinguished in a CEA sandwich immunoassay by varying the buffer components and consequently the PK digestion conditions. In other words, the Prionics buffer may be influencing cleavage of PrPres such that the epitopes to which 6H4 and P4 bind are affected differently when digested in PK. Further investigation is required. The known sheep isolate CH1641 behaves similarly to BSE in ARQ/ARQ sheep with respect to differential staining, but can be distinguished by glycoform differences.

b) <u>PrP^{Sc} glycosylation and the ratio of glycoforms of the PrP^{Sc} fragments (Collinge *et al.*, 1996; Somerville *et al*., 1997; Kuczius *et al.*, 1998):</u>

Following digestion of PrPres in Proteinase K, detection of residual PrP after Western Blotting generally reveals three protein bands. The upper band is recognised to carry two sugar molecules (diglycosylated), the middle band one sugar molecule (monoglycosylated) while the third and lowest is unglycosylated. It was found that the banding pattern and ratios of intensity of the bands conformed with clinical and pathological phenotypes of forms of CJD (Parchi *et al*, 1996). More importantly, the banding patterns for vCJD and BSE sources were indistinguishable, as were those of other BSE derived isolates, such as FSE (Collinge *et al* 1996). The lower molecular weight of the unglycosylated band, and the ratio of di to monoglycosylated bands (with diglycosylated band density being greater than monoglycosylated), were potentially diagnostic of BSE. This was not however tested on known BSE infected sheep at the time, and the number of BSE-infected sheep on which it can be verified still remains small, and limited to sheep of certain genotypes (ARQ/ARQ; AHQ/AHQ). The glycosylation “fingerprints” in other genotypes of sheep remain to be determined.

While a high level of diglycosylated PrP^{Sc} has been shown to clearly distinguish vCJD from other forms of TSE in humans (Collinge *et al*, 1996) and in mice, BSE also gives a high level of diglycosylated protein. Also it does not allow BSE sources to be distinguished from all mouse-adapted scrapie strains (Somerville, 1997b). Some studies have failed to find a statistically significant difference in this respect between BSE in cattle and natural scrapie cases in sheep, but have revealed a uniform PrP^{Sc} pattern in sheep (Baron *et al*, 1999; Stack *et al*, in press). A study of sixteen Irish sheep scrapie cases also showed similar and uniform patterns in sheep (Sweeney *et al*, 2000), but also, according to the antibodies used, some differences between scrapie in sheep and BSE in cattle. These results however do not exclude that differences may be linked to species differences rather than to strain differences in the antibody reactivities. Another study also showed, using mouse antibodies produced against recombinant ovine prion protein, that major differences in the glycoform ratios of sheep and cattle PrP res could be found using different antibodies (Betemps *et al*, 2001).

c) The relative proteinase resistance of PrP^{Sc}:

Under defined experimental conditions, PrP^{Sc} from different experimental BSE and scrapie strains/isolates, as well as field sources, display rather non-uniform degradation kinetics during prolonged proteinase K exposure. Residual PrP^{Sc} levels can be measured by quantitative immunoblot. Typically, after 6 h of exposure, residual PrP^{Sc} levels vary in a broad range of less than 20% to more than 80% of the respective reference value. While BSE PrP^{Sc} seems to be relatively PK sensitive, PrP^{Sc} of most scrapie strains exhibit a rather high resistance to prolonged exposure. This distinction was found for PrP^{Sc} of mouse adapted experimental BSE and scrapie strains as well as for ruminant derived PrP^{Sc} (including scrapie and BSE PrP^{Sc} from ARQ sheep) (Kuczius and Groschup, 1999).

d) Molecular conformation of PrP^{Sc}: An approach based on an immunoassay quantifying the binding of an antibody against the denatured/native forms of PrP^{Sc} has been proposed (Safar et al., 1998). Combining the ratios of binding against these two forms of PrP^{Sc} and the concentration of PrP^{Sc}, different features have been shown in eight different hamster adapted scrapie strains.

The method relies on the principle of the "conformation dependent immunoassay (CDI)" which has been submitted to the EC for evaluation as a rapid test for the diagnosis of BSE in bovines. The CDI is based upon measuring the immunoreactivity of an epitope in PrP that is exposed in PrP^{C} but buried in PrP^{Sc}. Because the CDI does not require protease digestion to remove PrP^{C}, both protease sensitive and protease resistant PrP^{Sc} are measured. According to Richard D.Murdock (pers.comm, 9 January 2002), it seems likely that the CDI will be able to distinguish a variety of sheep scrapie sources from each other and from sources of BSE in sheep. Such an approach could thus be developed to distinguish BSE and scrapie sources/isolates. It must be emphasised that no data obtained with BSE, and particularly BSE in sheep or goats, has ever been described using this approach.

II.2.3. Tissues to be used for molecular typing.

It is important to point out that the above listed molecular typing techniques are only of use, at the moment, at *post mortem* as they are designed for utilisation of brain samples from clinically affected animals. Provided that the differences in molecular typing patterns between BSE and scrapie are maintained on application of the methods to peripheral (lympho-reticular) tissues, the techniques might be used to advantage for early preclinical differentiation of BSE and scrapie. Western Blot and similar PrP^{Sc} techniques are presently being investigated to look at pre-clinical stages and at peripheral tissues which might be useful for a pre-mortem test. However, the peripheral responses of sheep differ according to PrP genotype (and possibly also the infecting strain of agent) as to whether they have LRS PrP accumulation or not. Unless information on the pathogenesis is available for a particular genotype and breed with regard to LRS PrP accumulation during incubation, ante-mortem biopsy methods may provide false negative results.

II.2.4. Pathological approaches to phenotype definition and differentiation in the host species

As used in strain typing with mouse models of scrapie, described above, the neuropathology (lesion profile) of a TSE in a particular genotype of host can give information on the source of the disease. Characterisation of host pathology outwith the experimental situation can also inform on the source of the disease. This approach has been widely used in the phenotypic characterisation and definition of human forms of TSE and other prion disorders. In the strictly controlled mouse models of scrapie a method of scoring the intensity of vacuolation in selected brain areas at the late clinical stage of disease has provided the “lesion profile” which is a characteristic “fingerprint” of that model if all experimental conditions are constant. The profile is influenced by several factors, most important of which are agent "strain" and host genotype. The molecular basis of agent strain is not yet understood, but host genotype is determined principally by the PrP gene.

Clearly, the lesion profiles of naturally occurring TSE in animal populations are uninterpretable where host and agent factors show variation and this has certainly been the experience in the past with natural sheep scrapie. In sheep it has not so far been possible to recognise strains using characterisation of the vacuolar pathology in the brains of affected sheep.

In contrast to that of natural scrapie, the lesion profile of BSE in cattle is remarkably uniform, mimicking the stereotypy seen in controlled experimental models of scrapie. Lesion profiling has been applied to define the vacuolar pattern in BSE (Wells et al., 1989, 1992, 1994, 1995; Simmons et al., 1996). From this and other evidence it has been proposed that the major factors controlling expression of the lesions are constant. The implication that the epidemic has been due to a single, stable, cattle adapted strain of a scrapie-like agent is supported by the uniformity of the phenotype of disease on primary transmission of BSE to mice and by subsequent strain typing. A similar approach has been adopted to demonstrate the similarity of the BSE phenotype in cattle occurring in Portugal (Orge et al., 2000).

Profiling of vacuolar changes in the brains of cattle with BSE has been used to monitor the pathology of the current epidemic in Britain over time. No evidence of phenotypic differences in the disease was detected in the period 1992-1995

(Simmons et al. 1996), or subsequently (Simmons et al unpublished data), adding further support to the notion that the epidemic has been sustained by a single agent strain. Uniformity of the lesion profile also in Feline Spongiform Encephalopathy (FSE) (Wells et al. 1994 and unpublished) complements the results of primary transmissions of FSE and BSE to mice suggesting that FSE is the consequence of exposure of domestic cats to the BSE agent.

There are now two studies (Ligios et al., in press, and Begara-McGorum et al., in press) which address the pathological phenotype of natural scrapie, based upon vacuolar lesion profiling. These conclude that the approach may assist in identifying a particular disease phenotype within a flock, but due to the variation provided by several host and agent factors, is probably limited as a means of discriminating between natural scrapie strains.

The accumulation and neuroanatomical targeting within the CNS of the pathological isoform of the protein, PrP, is considered to be controlled similarly to that of the targeting of vacuolation. The detailed topographical and cellular localisation of PrP accumulation and its relationship to other morphological changes can be shown immunohistochemically. In BSE distinctive morphological configurations of reactivity have been described (Wells and Wilesmith 1995, Orge et al., 2000). Particular immunolabelling patterns characterise certain neuronal groups. Consistent with studies of experimental scrapie in rodents the topography of immunolabelling may contribute additional information to the lesion profile.

Patterns of PrP accumulation in natural scrapie may offer a more promising approach to the identification of sheep scrapie strains than phenotypic characterisation using vacuolar profiles (Gonzalez *et al.*, 2002). Different cellular and neuroanatomical types of disease-specific prion protein (PrP^d) accumulation are found in the brain of scrapie affected sheep of different breeds and PrP genotypes. These types of PrP^d accumulation may be scored and arranged into patterns or profiles. Analysis of the differences in magnitude and relative proportion of each of these PrP^d types and patterns have indicated that there is an effect of the scrapie source on the PrP^d profile. Differentiation of at least some scrapie sources was possible by PrP immunohistochemical examination of brains of affected animals when the analysis was conducted on a group basis (groups being defined by breed and PrP genotype). The extent as yet to which this differentiation can be attributed to the major factors (agent strain and host genotype) controlling disease phenotype requires further work. Only a limited range of natural sheep scrapie sources has so far been examined using this system.

Jeffrey *et al.* (2001) have described a provisional immunohistochemistry approach using PrP antibodies directed at certain peptides of the protein which is able to discriminate between sheep scrapie sources and BSE infection of ARQ/ARQ genotype sheep (including discrimination within the Lympho-Reticular System). In extension to this work (unpublished) Jeffrey *et al* have combined their peptide mapping approach – see below - (Jeffrey *et al.*, 2001) with the PrP profiling and shown (Jeffrey, pers.comm, 5.02.02) that there appears to be multiple naturally occurring sheep scrapie sources, but quite how these phenotypic differences reflect strain diversity is, as yet, unclear. This approach requires to be replicated. So far, as with other potential discriminatory methods, it has not been applied to experimental BSE of sheep with VRQ/ARQ or

VRQ/VRQ genotypes. It remains to be seen also how some scrapie isolates, such as CH1641, which share biochemical properties with BSE regarding their cleavage sites by proteinase K, behave using such an approach.

Such an approach would require clinical cases and whole, or sagitally cut hemi-brains **and/or selected lymphoid tissues** fixed in formalin.

II.3. THE POSSIBLE APPLICATION OF BSE-IN-CATTLE RAPID TESTS FOR TSE DETECTION IN SMALL RUMINANTS.

The three rapid TSE tests as currently applied to cattle are considered to be reliable detectors of BSE PrP^{Sc} in cattle brain material in the clinical stages of disease and in late incubation (Moynagh and Schimmel 1999).

5 new rapid TSE in cattle tests are currently being evaluated by Commission Services. In the development phase most if not all of these tests have been applied to scrapie sheep brains because they were more readily available for preliminary work. Due to the similarity of the brain matrices and the non-specificity of the antibodies used it is anticipated that these tests will be effective for detection of BSE PrP^{Sc} in sheep. Commission services would need to set up an evaluation programme to verify this.

Theoretically, at least, it should be possible to apply these tests to other lympho-reticular (LRS) tissues, e.g., lymph nodes or spleen. However, only some of the rapid tests have been applied to LRS tissue and on very limited numbers of samples. Since some of the tests are influenced by sample quality and history, even when confined to CNS tissue analysis, and changes of the sample treatment protocols (homogenisation and digestion/denaturation) are to be expected for the use of LRS tissues, an evaluation programme is definitely required. Also, it must be borne in mind that all of these tests detect PrP and their application to sheep LRS will therefore be limited to those sheep in which PrP accumulation in LRS can be detected.

III. CONCLUSIONS AND RECOMMENDATIONS

III.1. PROTOCOL FOR INVESTIGATIVE METHODS

It is unlikely that there exists within every country a laboratory that would be sufficiently equipped and with the appropriate experience to perform the second and third stage investigations. The Working Group therefore recommends that, for the context concerning the EU, those national laboratories that are able to perform the second and third stage investigations are identified and they then analyse the positive samples received from other countries. Taking into account its experience and equipment, each such laboratory should identify for its use at least 2 from the molecular methods listed in section II.2.2. The same or additional laboratories able to perform biological strain typing, as in section II.2.1, need also to be identified.

As explained elsewhere in this report, the outcome of a single molecular typing approach will not provide proof beyond any doubt that BSE is present or excluded. Given their current state of development[8] it is also not yet possible to

[8] Which implies also that comparability of results between laboratories has not been verified and that appropriate availability of standardised reagents and antibodies is not necessarily guaranteed.

issue strong guidelines on which methods to use or which discriminatory thresholds to apply. Should the results obtained in a given laboratory provide indications that the TSE agent has a BSE-like characteristic, then they should be evaluated by an expert panel including experts from the various laboratories involved in the second stage of the investigation, from other national laboratories, from the Joint Research Centre and if appropriate, from other scientific bodies. Similarly, subsequent biological strain typing results should be reviewed by such a panel.

In the light of the above uncertain state of the art, the following general schema is suggested for the investigation as to whether BSE is present in the small ruminant population in the Community:

1) **First stage** (As soon as BSE-in-cattle rapid tests have been validated and adapted for use in small ruminants:) TSE surveillance performed on a statistically representative sample[9], using one of the currently available rapid tests for TSE in cattle, to identify animals affected with a TSE**.**

2) **Second stage** Investigation of the material from rapid test positive cases using the application of several (at least two) molecular methods listed in section II.2.3. of this report. (It should be noted that as other tests and methods become available the priority of application of methods may change.) This approach should be adopted only after interlaboratory validation of selected tests.

   The nature of PrP^{Sc} distribution in the brain means that careful consideration would need to be given prospectively to which combination of approaches will be used. This will have an effect on sampling strategy. If too many approaches are required in combination, the availability of sufficient tissue may prevent any successful conclusion. Sheep brainstems are small and the choice of approaches needs thus to be pragmatic, and based on the limited amount of optimal tissue available.

   Note: Before the above regime could be instituted on a routine basis, the following needs to be done:

   a) The tests methodologies reported on so far in the literature and listed in Section II.2.2. should be confirmed by other laboratories. To some extent this has already been accomplished. For example WB results have already been reproduced, if we consider that they are only different tests based on the same principle. The work of VLA is one of several possible WB approaches, but the results are quite similar to those initially reported (Hill et al., 1998, Baron *et al.,* 2000), using two different WB methods. Comparison of the WB method of Baron et al (using ultra-centrifugation) with that described by Collinge in 1996 showed some differences in the electrophoretic profiles between the two methods, but the differences between BSE and natural scrapie, using a given method, are unchanged irrespective of which method is used.

      Furthermore, the development of the Prionics test proposed by the VLA for use in sheep is based on the same principles as those previously

[9] See also the Opinion of 29-30 November 2001 of the Scientific Steering Committee on the requirements for statistically authoritative BSE/TSE surveys.

described in mice by Baron and Biacabe (2001) using the SAF 15 antibody from C.E.A. Other antibodies directed against the same PrP region also give the same results (Baron, personal communication). The Elisa test developed by C.E.A. is also based on a similar approach. In each case an antibody that recognises an epitope which is deleted in BSE, but not in scrapie, has been applied.

Finally some methods could be developed as a version of a commercial test. However, it is emphasised that discrimination attempted on the basis of such tests would be required to be kept in the hands of specialised laboratories for the foreseeable future. Importantly, the tests require a BSE control, and would ideally require a control with BSE in sheep since, BSE in sheep is not identical to BSE in cattle from the molecular point of view.

b) For certain tests the availability of key antibodies will need to be ensured for long term continuity and EU standardisation of techniques. (e.g., the key antibody originating from the Federal Research Centre for Virus Diseases, Tübingen, Germany and used in the UK Veterinary Laboratories Agency tests described in II.2.2.). Better recognition of the biological phenomena that are generated by the current results may also indicate a need for improved, more specific, antibodies.

3) **Third stage**. Until current research on molecular methods of strain discrimination has yielded validated tests that allow differentiation on a routine basis, surveillance samples which are considered to provide results indicating a BSE-like characteristic, will need to be examined by biological strain-typing in mice as specified in Annex with the express objective initially, at least, of determining if a BSE-like strain is indicated on primary transmission. As indicated above, this method has been developed for application to aseptically sampled, high titre material from clinical cases. However, in as much as brain material identified positive by rapid tests will be from healthy slaughtered animals in the late stages of incubation, concerns regarding application of molecular typing and biological strain typing to preclinical material may not be critical. If results from the first and second stages indicate that the samples are unsatisfactory for the biological strain typing protocol proposed some consideration may need to be given to obtaining further material from suspect clinical cases of TSE in small ruminants. Given some uncertainties regarding the application of both molecular typing and biological strain typing to pre-clinical material this is a course of action which may need to be reviewed at the same time as any review of molecular typing results (see III.1.2 [Second stage]).

In conjunction with identification if a case of TSE in small ruminants with BSE-like characteristics on molecular typing, further investigation of the flock / geographic region of origin of the case should be conducted. This should include additional testing of clinical cases and epidemiological investigation (history of TSE occurrence, feeding practices etc.). Pending the outcome of mouse transmissions, the presence of BSE in small ruminants should be considered as being probable and the geographical extent of the risk would need to be assessed (i.e., EU-wide or confined to a country, region, flock or single animal). The Working Group notes that the Scientific Steering Committee is currently preparing an opinion on this matter.

4) Note:

The systematic and wide-scale use of rapid tests on slaughter populations to identify TSE infected animals followed by differential testing for BSE is likely to result in the second stage testing of very large numbers of animals and may not be the most efficient way to detect BSE. In order to optimise the use of resources, it is reasonable to propose to target the survey at areas and flocks/populations where the risk/likelihood of finding BSE, if it is present, is the highest regardless of the current (small ruminant-) TSE incidence in that area or flock. This could well be, particularly in the first instance, areas or countries where BSE is present or where the BSE risk is highest. In the report attached to the SSC's Pre-emptive risk assessment on BSE in sheep of 8-9 February 2001, the factors are listed that should be taken into account when assessing the likely future prevalence of BSE in sheep. They could be used as a tool for identifying target areas and populations. Individual flocks with particularly high risk factors could perhaps be targeted, e.g. those which used supplementary feeding extensively during the period of highest risk, and have subsequently not diluted the flock substantially by purchase of stock. The results obtained by such targeting could be very valuable even if the result is negative (implying that 'high risk' practices did not result in disease, or at least that infection is difficult to sustain in a flock over the intervening period).

III.2. SAMPLE SELECTION

BSE in small ruminants, should it occur, is likely to represent only a fraction of scrapie prevalence. The SSC opinion on *Requirements for a statistically authoritative TSE survey*, adopted on 30 November 2001, provides guidance for the methods for sample selection and number of samples to be analysed per country. It calculates that a country would need to apply second-stage BSE testing to between 600 (95%) and 920 (99%) TSE rapid test positives to find at least 1 BSE case should the BSE prevalence be 1 per 200 TSE test positives. To find at least 1 BSE case if the BSE/TSE ratio is 1 in 2000, between 6000 (95%) and 9200 (99%) TSE rapid test positives would need to be tested.

What precedes does not mean that such numbers imperatively need to be submitted to second stage testing, as the number can of course not exceed the number of positives found in the first stage. However, given the relatively low incidence (in statistical terms) of TSEs, it is recommended that all animals found positive in the first stage, are also submitted to the second stage testing. The confidence that BSE is not present in small ruminants will increase with the numbers of animals that tested negative in the second stage.

III.3. OTHER RELEVANT ISSUES

III.3.1. Pooling of samples

The above approach is likely to lead to very large numbers of samples from individual animals to be submitted for TSE testing and possibly to a subsequent step aimed at strain differentiation. However, the pooling of individual samples is for the time being an unworkable solution to reduce the required resources, for the reasons explained hereafter.

a) Pooling of materials from several TSE suspect animals, before the rapid test step would result in a dilution of the infectivity in the material. D*ue to the*

existence of samples with a low concentration of the analyte and the dilution by pooling the risk of creating false negatives is significant and therefore pooling cannot be recommended. Moreover, the recent evaluation exercise of 5 new rapid TSE tests has shown (IRMM, unpublished) that the homogenisation of material that would be a requisite of pooling, may severely affect the efficacy and reliability of the rapid tests (and possibly of other molecular tests as well). Inappropriate homogenisation techniques can either reduce the signal specifically for some tests or in the worst case all infectivity or signal irrespective of the test applied.

b) With regard to molecular methods, it has clearly been shown that, at least in some situations, the recognition of BSE was not possible in a mixture of both BSE and scrapie (Baron *et al,* 2001).

Pooling of purified SAF preparations of murine passaged scrapie and BSE agents has shown that the scrapie strain masks detection of the BSE strain, making detection of BSE when combined with scrapie impossible, using western blotting to assess molecular weight (Baron *et al*, 2001)

It has been suggested that when a mixture of strains is strain-typed in mice the lesion profile can be used to identify which strain results in the death of the mice (Bruce, 1996) i.e. the strain with the shortest incubation period. The experience of mixed inocula is that (M.Bruce, pers.comm, 28 February 2002), if the incubation periods of the two strains are not that different there is the potential for a hybrid profile, which would be a problem. There are experiments underway looking at strain typing of mixed BSE/sheep scrapie inocula, which should help, but the results are not yet available. It may thus be unlikely that primary transmission of a pooled sample would give interpretable results, but that a single further passage from VM mice would be necessary to look for 301V characteristics. This could suggest that if more than one strain co-existed in a sheep (eg BSE arising in a flock with endemic scrapie) the strain which develops into clinical disease in a sheep may mask the presence of another one, which could still present a health risk, e.g. incubating scrapie might mask the presence of BSE in a sheep, but the 'relative pathogenicity' of the strains may change on passage to another host.

Ongoing work at the UK Veterinary Laboratories Agency (VLA) seems further to indicate that transmission from a pool (in terms of producing clinical disease) may result in only a minority of scrapie sources transmitting well to mice and selection from these may not be representative of strains causing disease in the sheep making up the pool.

In view of the above concerns, and the absence of any direct evidence that the BSE agent can be isolated from a pool containing an excess of scrapie agents, the use of pooled inocula for the identification of BSE is unwise.

III.3.2. Collection and storage of tissue samples

A disposable plastic spoon (modified spatula) for the removal of the ovine brain stem has been developed at VLA and is currently used for collecting samples for routine testing of sheep for TSE. It is imperative that collection should ensure that there is no risk of cross-contamination with BSE of bovine origin, or TSE material from a separate ovine source and that samples are collected aseptically. Under the conditions of the abattoir thus should be achievable by the use of fresh disposable instruments for each animal.

Note:

Once molecular rapid testing and typing methods are fully developed and validated for use in lymphoid tissue, they can be applied to lymph nodes (submandibular and medial retropharyngeal) and tonsil which are readily accessible post-mortem. For this eventuality, these tissues need to be collected in surveys from the outset.

It is unlikely that it would be possible to ensure adequate identification of all small ruminants at slaughter and thereby conduct PrP genotyping on blood samples taken on farm. In this case genotyping could be performed on fresh brain tissue, but this inevitably reduces the amount of tissue available for molecular methods.

The fresh brainstem should be dissected to provide: 1) A slice –approximately 1cm in length – inclusive of the obex region, into formalin, for confirmatory examinations [histopathology and PrP immunohistochemistry]; 2) medulla, caudal to the obex and any cranial spinal cord, to be frozen [-70 to -80°C, or -20°C, depending on availability], for molecular methods; 3) All remaining fresh tissue, rostral brainstem etc. to be stored [-70 to -80°C, or -20°C, depending on availability] for subsequent strain typing (and possibly for genotyping).

If lymphoid tissues are to be collected also, one portion of each tissue should be fixed in formalin for PrP immunohistochemistry and the remaining stored frozen for molecular methods.

Notes:

No quantitative data on the distribution of PrP^{Sc} in CNS tissue of sheep are available. From the data obtained in the BSE test evaluations carried out in 1999 and 2001 one can deduce that in clinical BSE cases in cattle the PrP^{Sc} concentration in brain stem is higher than in spinal cord and that on average the maximum concentration is located at or slightly rostral to the obex region (the obex samples were not available and can only be interpolated from between the regions caudal and cranial to the obex). It should be noted that there is a large spread of test readings in one region and therefore that e.g. the obex region has always the highest PrP^{Sc} level in the brain stem of one individual. Whether or not this observation is true for sheep scrapie has not been determined. Qualitative observations on immunohistochemistry of natural scrapie in the UK suggest that both the total amount of PrP and its distribution within the brainstem is much more variable than in BSE (M Simmons, personal observations, 2002)

The amount of material sampled initially for testing is important. Taking small sub-samples bears the risk of creating false negatives (based on data from bovine brains). An initial minimum sample size of 0.5 to 1 g for molecular test gives the possibility for repeat testing where necessary. If the option of applying several tests and retaining reserve material is to be available it is recommended to sample the whole brain stem (as described) or obtain a total weight of brain stem of not less than 10 – 15 g). Each aliquot of fresh material should be taken as a complete, or sagitally divided transverse slice through the neuraxis. Freeze drying of the samples should be avoided, i.e. the containment should not have too much head space and be sealed. The temperature cycles of

the freezer should be as low as possible. The impact of prolonged storage of homogenates or repeated freezing / thawing cycles on some of the rapid tests is not completely clear but effects have been observed and are more pronounced in homogenates compared to tissue samples.

It cannot be excluded (only oral communications) that samples already containing low PrP^{Sc} concentrations are more strongly influenced by autolysis or storage than samples containing high PrP^{Sc} levels. Therefore also for autolysed samples appropriate and systematic sub-sampling as well as direct comparison with other tests on equivalent sample sets is required in order to be able to draw valid conclusions.

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ANNEX: THE FAIR 5 - CT97 3305 PROTOCOL FOR SCRAPIE ISOLATE PHENOTYPING USING BIOASSAY IN IN-BRED MOUSE LINES.

A protocol for scrapie isolate phenotyping using bioassay in in-bred mouse lines was agreed upon on March 6th, 1998 as part of the EU sponsored The FAIR 5 - CT97 3305 research project. The scientists involved were: Moira Bruce, Michael Dawson, Jean-Michel Elsen, Wilfred Goldmann, Martin Groschup Nora Hunter, Frédéric Lantier, Pierre Sarradin and Torres Sweeney. The protocol is as follows:

SAMPLE PREPARATION:

Samples collected aseptically for bioassay should be stored frozen at -20°C, or lower. Repeated freeze-thawing cycles should be avoided. All containers and instruments used for the storage and transfer of tissues should be new, autoclaved before use and discarded after use.

Samples for inoculation should be macerated with sterile, disposable scalpels and homogenised in sterile Griffiths tubes or Dounce homogenisers, at 10% w/v concentration in sterile physiological saline. (Note it is imperative that new, autoclaved, homogenisers are used for sample preparation.)

The suspension should not be clarified by centrifugation, and ideally antibiotics should not be added, nor should the inoculum be heat treated. Reserve aliquots of inoculum and brain material should be stored at -20°C, or lower, in new sterilised glass containers.

INJECTION OF MOUSE PANEL:

Inoculum from each sheep source should be injected into three inbred mouse strains - RIII, C57BL and VM. Breeding stock to produce experimental mice must originate from the Neuropathogenesis Unit (NPU) of the Institute for Animal Health at Edinburgh (United Kingdom). At least 20 mice of each strain should be inoculated with each inoculum. Each anaesthetised mouse should be inoculated with 20µl by the intracerebral route and 100µl by the intraperitoneal route.

At least one full panel of mice should be inoculated with saline to provide controls for clinical and pathological studies, and at least one full panel should be inoculated with BSE to represent a standard TSE isolate known to transmit to all mouse lines.

CLINICAL SCORING OF MICE (BASED ON SYSTEM DESCRIBED BY DICKINSON ET AL., 1968):

Animals should be routinely monitored for signs of intercurrent disease throughout the experiment. Additionally, at 250 days after injection, a formal clinical monitoring system should be started. Animals should then be checked daily and scored weekly (on the same day each week) for neurological signs, indicative of TSE disease, by trained observers. Animals should be classified as being "unaffected", "possibly affected" or "definitely affected".

Animals should be sacrificed a) after two consecutive weekly scores of "definitely affected", b) after receiving scores of "definitely affected" in two out of three consecutive weeks or c) if there is a significant deterioration between scoring days, beyond the stage recognised as "definitely affected", irrespective of the actual score on the preceding scoring day. For example, animals should be killed as soon as the clinical disease progresses to include any signs of paralysis of the hind limbs or an inability to feed or drink.

COLLECTION OF BRAINS FROM THE MICE:

Brains should be collected from all mice surviving beyond 100 days after challenge, including those sacrificed with intercurrent disease unrelated to TSE infection. Each brain should be dissected using new, autoclaved, disposable instruments. A lateral third of each brain should be collected for further transmission and/or biochemical analysis. This involves cutting the brain, with a sterile disposable blade, along a parasagital line about halfway between the midline and the lateral extremity of the cerebral cortex on the upper surface of the brain, with the cut tapering in towards the midline on the lower surface of the brain. The smaller part should be frozen unhomogenised in sterilised containers at -20°C or lower. Individual mouse spleens should also be collected aseptically. The larger sample of each brain should be immersion fixed in 10% formol saline.

LESION PROFILING (FRASER & DICKINSON, 1968):

Formol-fixed brains should be trimmed coronally at 4 standard levels, according to a protocol held at NPU. The brain slices should be paraffin embedded, and 6µm sections stained with H&E. Coded brain sections should be examined and vacuolar lesions, indicative of TSE disease, should be scored on a scale of 0-5 in nine standard grey matter areas and on a scale of 0-3 in three standard white matter areas, as specified in protocols held at NPU.

DATA ANALYSIS:

For mice with clinical TSE signs and a histopathological confirmation of TSE disease, the incubation period should be calculated as the interval in days between challenge and death. For each challenge experiment, the mean incubation period for each mouse strain should be calculated. Lesion profiles should also be constructed from the mean vacuolar lesion score in each brain scoring area. The results of a range of transmissions may be compared using hierarchical cluster analyses that are currently being developed.)

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