UNION EUROPÉENNE

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Object: Manual of Standards – EC Comments – May 2007

Dear Bernard,

Please find attached as an annex to this letter the Community comments on the "4th mailing" of the Biological Standards Commission, with reference to certain modifications of the Chapters in the OIE Manual of Standards and Vaccines, which should be adopted at the next General Session.
The comments have been gathered in the format as required. Some of these comments have already been sent directly by some of our laboratories, which are OIE reference laboratories. Nevertheless, some of the comments seem out of date, as in the meantime further meetings of specialists have taken place, and the versions modified in consequence, without comments by the Member countries.
In this context, the Community would ask the OIE to provide the revised 4th version of the draft chapters.
Thank you for the continued excellent collaboration and trust you will find our comments constructive and useful.

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ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 1.1.6. Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities

Country making the comments: European Community

Date

General Comments

In the introduction and Chapter A in particular there is a need to include spread into the environment, which is more than just causing disease in animals. A number of other track changes are made but these are largely for clarification of the text.

Specific Comments (add continuation sheets if required)

Line 2: There is a need to provide a definition of both biosafety and biosecurity - these may be defined elsewhere in OIE documentation so needs to be consistent with those definitions, or cross reference to them.

Line 8/9: The end of the second sentence of the paragraph should read: "measures to minimise the risk both of human disease and of possible release into the environment."

Line 14/15: The sentence should read: "One is to prevent disease in humans; the other is to prevent the release of the pathogen into the environment and so causing disease in animals."

Line 21/23: The sentence should read: "When new laboratories are being established, it would be prudent to seek advice from the relevant regulatory authorities and competent authorities at established institutes."

Line 39: The sentence should begin by "Known occurrence of human and animal infection".

Line 45: Same remark "may contain human and animal pathogens".

Line 78: "Additional" should be replaced by "Different"

Line 85: The second sentence of the paragraph should read "Some examples of the pathogens that may cause disease in humans, and also be found in a veterinary laboratory are listed in Table 1."

Line 95: The words "and the environment" should beaded at the end of the first sentence.

Line 98: It should be a "Biological Safety Officer".

Line 143: Class I, II or III can be used at containment level 2. The important point is that they are used whenever a procedure is being used that might generate an aerosol. The sentence should more simply read "Containment level for Group 2 pathogens, in addition to the points given above, a Class I, II or III microbiological safety cabinet should be used when there is the potential for generating aerosols."

Line 151: The words "qualified level 3" should be replaced by the words "appropriately trained".

Line 162: The safety cabinet should not just be provided, but guidance given as to the circumstances in which it should be used; the words "and used whenever the process to be undertaken is likely to generate an aerosol" should be added at the end of the sentence.

Line 256: The word "autoclaving" should added before the words "steam sterilisation".

Line 287: The first sentence should read "Great care must be taken when preparing and packing diagnostic specimens, infectious materials and pathogens for transport,… etc."

Line 342/343: The first sentence should end by "into the environment and the national animal population".

Line 347: There is a need to be more specific; the words "related to" should be replaced by the words "not less than".

Line 355: Other experimental animals may be used. At the end of this paragraph, the following sentence should be added: "In addition, other animals being used for experimental work on the pathogen should be held in the appropriate containment level."

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.2.7. Heartwater

Country making the comments: European Community

Date

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

No specific comment.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.2.8. New world screwworm (*Cochliomyia hominivorax*) and old world screwworm (*Chrysomya bezziana*)

Country making the comments: European Community

Date

General Comments

No comment.

Specific Comments (add continuation sheets if required)

No specific comment.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.2.12. Rinderpest

Country making the comments: European Community

Date

General Comments

Some of the additions to the text are not relevant and/or not (yet) proven enough. See below.

Specific Comments (add continuation sheets if required)

Line 194: The whole footnote (1) should be reintegrated within the text at the end of the paragraph.

Line 236: Same comment, the whole footnote (2) should be placed between the words "the published procedure." and the words "The resulting RNA".

Lines 283/284: The sentence "The antigen is concentrated from the infected cell culture supernate by ammonium sulphate precipitation" should be deleted.

Lines 315/323: The whole paragraph ix) should be deleted, as well as the next paragraph.

Lines 356/362: This whole part that was added should be removed.

Line 367: The words "future use appears unlikely even for the control of PPR in small ruminants" should be removed.

Lines 507/509:The reference 17is irrelevant and should be deleted.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.2.18. *Trypanosoma evansi* infections (including surra)

Country making the comments: European Community

Date

General Comments

No comment.

Specific Comments (add continuation sheets if required)

No specific comment.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.2.19. West Nile fever

Country making the comments: European Community

Date

<u>General Comments</u>

The reagent manufacturers should be named in the Manual of Standards.

<u>Specific Comments (add continuation sheets if required)</u>

Line 23: besides nested RT-PCR, real-time RT-PCR assays have been developed for WNV. Quantitative PCR should be more developed, as it is more used.

Line 42: lineage 2 virus also reported from Hungary

Lines 51-53: the equine WNV outbreak in Argentina 2006 should be added

Line 60: Differential diagnosis in horses include …..

Lines 104/105: Tewari et al. (2004 ; J. Clin. Virol. 30, pp. 320-325) described the use of a real-time RT-PCR for equine tissue. Niedrig et al. (2006; Clin. Chem. 52, pp. 1851-1854) performed the first International proficiency study on West Nile virus molecular detection.

Line 171: Add a dot before “Include…..”

Lines 184/185, the sentence should be: "Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), hemagglutination inhibition (HI), IgG ELISA or plaque reduction neutralisation (PRN) (2, 12)".

Line 192: …. will be encountered.

Lines 199, 206, 222: “normal antigens” (lines 199, 222) and “control antigen” (line 206) seems to be the same and might be better termed “negative control antigen”

Line 255: In 2004, an inactivated human cell line-derived WNV vaccine developed by Crucell NV (The Netherlands) and Kimron Veterinary Institute (Israel) has got a market authorization in Israel as a veterinary vaccine for geese.

Lines 290/291: perhaps better to say: “The DNA vaccine expression cassette is amplified in *E. coli* using a plasmid vector, cutted out from the plasmid backbone and purified for formulation into a vaccine.”

Line 296: What could be the highest level of LPS contamination of the DNA vaccine?

Lines 320/321: What is about testing the potential LPS contamination? What is meant by “antigen quantification” in terms of a DNA vaccine?

References:

Tewari D, Kim H, Feria W, Russo B, Acland H. (2004). Detection of West Nile virus using formalin fixed paraffin embedded tissues in crows and horses: quantification of viral transcripts by real-time RT-PCR. J Clin Virol. 30, 320-325.

Niedrig M, Linke S, Zeller H, Drosten C. (2006). First international proficiency study on West Nile virus molecular detection. Clin Chem. 52, 1851-1854.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.3.13. Bovine spongiform encephalopathy

Country making the comments: European Community

Date

General Comments

This Chapter should be the universal collection of methods that should be applied for the screening and confirmation of BSE cases in animals. To fulfil this purpose, it must be precise and comprehensive. This goal has not in all points been achieved in the present draft version. Moreover, it must be noted that most NRLs worldwide, if not all (including the four OIE reference laboratories), use the state-of-the-art immunohistochemical and biochemical techniques. Therefore the current draft version should be amended.

Specific Comments (add continuation sheets if required)

As the specific comments were too important to be detailed here, please find attached an amended version with track changes.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.4.1. Ovine epididymitis (*Brucella ovis*)

Country making the comments: European Community

Date

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

Comments have been already made by the specialists directly to the OIE.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.4.2. Caprine and ovine brucellosis (excluding *Brucella ovis*)

Country making the comments: European Community

Date

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

Comments have been already made by the specialists directly to the OIE.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.4.7. Enzootic abortion of ewes (ovine chlamydiosis)

Country making the comments: European Community

Date

It would be appreciated if the following guidance notes are followed in making a reply:

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

Line 7: The words "the last 1-2 weeks of pregnancy" should be changed to "the last 2-3 weeks of pregnancy", which then is in agreement with line 48.

Line 76: The following sentence should be added: "Environmental contamination resulting from such sources is considered the primary source of infection to other females. It has also been shown that sheep that experienced an abortive episode following experimental infection excreted detectable amounts of chlamydial antigen from the reproductive tract during subsequent oestrus cycles (21)." And the sentence following should read: "Human infection may be acquired from infected products of abortion or parturition or from… etc."

Line 188: The following sentence should be added: "A western blot which examines for antibodies against specific antigens of purified elementary bodies has been used as a reference test on samples testing positive by CFT in a sheep health scheme (15)."

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.5.3. Equine encephalomyelitis (Eastern & Western)

Country making the comments: European Community

Date

General Comments

No comment.

Specific Comments (add continuation sheets if required)

Line 113: It is proposed to add: "Recently, a combination of a reverse transcription-PCR with an enzyme-linked immunosorbent assay (RT-PCR-ELISA) was developed to identify human pathogenic alpha-viruses (Wang et al., 2006)."

Lines 188/189: It is necessary to specify what is used as "control antigen".

Line 195: Instead of flasks, 6-well plates could be also used.

Line 199: "3.3% of a 1/1500 dilution of neutral red (1/8000)": the meaning of 1/8000 should be explained.

Line 216: Replace "good immunity" by "protective immunity".

Line 228: The terms "extraneous agents" should be specified.

Reference

Wang E, Paessler S, Aguilar PV, Carrara AS, Ni H, Greene IP, Weaver SC. (2006). Reverse transcription-PCR-enzyme-linked immunosorbent assay for rapid detection and differentiation of alphavirus infections. J Clin Microbiol. 44, 4000-4008.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.5.12. Venezuelan equine encephalomyelitis

Country making the comments: European Community

Date

General Comments

No comment.

Specific Comments (add continuation sheets if required)

No specific comment.

Lines 147, 153, and 158: There is no reference to ”[1]”.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.5.13. Epizootic lymphangitis

Country making the comments: European Community

Date

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

No specific comment.

Line227: the Ameni paper listed as "2005 in press" has been published - 2006 - Veterinary J., 172 (3):553-555

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.6.3. Teschovirus encephalomyelitis (previously Teschen/Talfan diseases)

Country making the comments: European Community

Date

General Comments

No major comment.

Specific Comments (add continuation sheets if required)

Lines 67-72: The typical clinical signs are more a flask paralysis than tremor and opisthotonos even if signs of encephalitis such as convulsions and nystagmus can be observed in young piglets (see publication: identification of two outbreaks of Talfan disease in Brittany by P Vannier and F Bernard, Rec. Med VET, 1977, 153, (10), 647-653.). The clinical signs described there are more those observed for Aujeszky's disease than for the more or less recent cases observed on Teschen disease. In consequence, this chapter is very confusing as there is no chapter on differential diagnosis with the diseases related to Teschen such as AD or *Streptococcus suis.* Such a chapter in 2.6.3 is absolutely needed.

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.6.8. Porcine reproductive and respiratory syndrome

Country making the comments: European Community

Date

General Comments

Whilst the level of detail provided on tests is useful to those trying to establish the test, it can sometimes be a hindrance to those performing tests under ISO17025, since it places an absolute requirement to perform such testing in strict adherence to the detailed protocol.

In many cases, this may not be possible, or experienced labs may refer subtle modifications that, for them, provide enhancements in sensitivity, reading etc, or alternative volumes for convenience, where large numbers of tests are performed.

Examples include the volume that harvested cells are suspended in, prior to counting, and Line 119, the exact vial volume and cell concentration (which is actually different from that stated for freezing down – line 102!). We would recommend insertion of "approximately" or "a suggested volume" …etc

Specific Comments (add continuation sheets if required)

Line 7: The words "newly established" should now be deleted. This Order has been established for some years.

Line 13: The word "sometimes" should now be replaced by "usually".". In field cases, uncomplicated respiratory disease due to PRRS alone is rare, if never.

Line 14: After the first two paragraphs, the following sentences should appear to complete before the paragraph on identification: "The virus is primarily transmitted via infected pigs, and also by faeces and urine. The virus can also be transmitted via semen and fomite spread via contaminated surfaces can also occur."

Line 15: It is proposed to replace "is" by "can be".

Line 30: The words "cell and" should be inserted between "both" and "PRRS".

Line 34: The word "also" should be added between "have" and "been".

Line 39: The word "potential" should be deleted.

Line 53: At the end of the first paragraph of the introduction, the following sentences should be added: "There is increasing diversity among strains of the two genotypes, which has been attributed both to the high error-rate inherent in PRRSV replication (Chang *et al,* 2002) and recombination between strains (VanVugt *et al,* 2001). There have also been recent descriptions of strains with high degree of polymorphism, providing further insights into the emergence of this relatively new

pathogen of pigs (Stadejek *et al,* 2006). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered."

The reason is self-explanatory.

Line 116: The last sentence of the paragraph should read: "After a period of 1-2 days or once a CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a specific antiserum or monoclonal antibody (MAb)." This is because many labs stain at the end of the culture period, without checking for CPE and also use a mab, such as SDOW17, rather than antiserum.

Line 132: The word "dummy" should be replaced by "dilution". Better for foreign readers.

Lines 142 144, 145, and 148: The words "or MAb" should be added after "antiserum".

Lines 163 to 165: The sentence should read: "The identification of European-type strains of PRRSV in the USA and Canada has also been reported (Fang et al 2004), but the prevalence of infection by such strains is not well documented."

Lines 216 and 218: Change “dummy” to “dilution”.

Line 228: Insert “Dilute rabbit ant-swine (or anti-mouse, if staining isolation plate with Mab)”

Line 403: The words "bovine viral diarrhoea virus" should be replaced by "all pestiviruses".

Line 480: The following sentence should be added at the end of this paragraph: "Likewise, there is mounting evidence that MLV vaccines of the European type can also spread to non-vaccinated animals and can also cross the placenta and give rise to congenitally infected piglets (Scortti *et al* 2006)".

The following References should be added:

To n°4: "Chang, C.C., Yoon, K.J., Zimmerman, J.J., Harmon, K.M., Dixon, P.M., Dvorak, C.M. & Murtaugh, M.P. (2002) Evolution of porcine reproductive and respiratory syndrome virus during sequential passages in pigs. *Journal of Virology* 76, 4750-63."

To n°8: "Fang, Y., Kim, D. Y., Ropp, S., Steen, P., Christopher-Hennings, J., Nelson, E. A., and Rowland, R. R. R. Heterogeneity in Nsp2 of European-like porcine reproductive and respiratory syndrome viruses isolated in the United States. *Virus Research* 100(2), 229-235. 2004."

To n°21: "Scortti, M., Prieto, C., Martinez-Lobo, F. J., Simarro, I. & Castro, J. M. (2006). Effects of two commercial European modified-live vaccines against porcine reproductive and respiratory syndrome viruses in pregnant gilts. *Vet J* 172, 506-14."

To n°22: "Stadejek, T., Oleksiewicz, M.B., Potapchuk, D. & Podgorska, K. (2006) Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. *J Gen Virol* 87, 1835-41."

To n°23: "VanVugt, J.J.F.A., Storgaard, T., Oleksiewicz, M.B. & Botner, A. (2001) High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. *Journal of General Virology* 82, 2615-20."

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.10.1. Cysticercosis

Country making the comments: European Community

Date

General Comments

This is an excellent description of the state of the art. It has been revised by a group of specialists.

Specific Comments (add continuation sheets if required)

Questions:

Line 129: AG-ELISA: Please include reference(s).

Lines 180 ff.: Such a test can hardly be validated if run as an in-house method. Who can supply control samples?

Specific changes:

Line 19: The sentence should read "Acute *T. multiceps* coenurosis and T. *hydatigena* cysticercosis in sheep and goats is rare but may be fatal."

Line 27: The word "diagnosis" should be replaced by "identification of species".

Line 58: The words "and pigs" should be added at the end of the sentence.

Line 59: The words "or necropsy" should be added at the end of the sentence.

Line 60: "viable metacestodes".

Line 62: The following sentence should be added "Eggs may also be disseminated by physical means or transport hosts."

Line 104/106 (and idem after): The word "carnivores" should be replaced by "canines".

Line 103: The words "(skeletal and cardiac)" should be inserted between "the musculature" and "of sheep".

Line 139: The words "provided food has been withheld for several hours (i.e. administer to dogs with empty stomachs)" should be added after the words "30 minutes".

Line 145: The following sentence should be added: "Keys for identification are given by Khalil *et al* (*)."

Line 193: Point ii) should read "Soluble aqueous extract of non-gravid proglottids from *Taenia* are obtained following emulsification in PBS and centrifugation."

Line 227: Add "tubular" before "long" and "wall".

Line 228: Add "as many as" before "85%".

Line 275: At point v), the words "usually after removal of the peritoneum," should be deleted.

Lines 330 to 333: The sentences before "Its presence or absence…" should be replaced by the following: "*T hydatigena* cysts usually mature in the omental or mesenteric fat but some may be retained at the liver surface where they are usually superficial and subserosal, while *Echinococcus granulosus* hydatid cysts tend to be deeper in the parenchyma. If viable, the former has a long-necked single scolex in a virtually translucent fluid filled cyst. Fertile hydatid cysts have thicker white outer membranous wall from which capsules bud and contain numerous protoscoleces which appear as a sandy deposit within the cysts. Differentiation can be important in the implementation and monitoring of hydatid disease control measuresfor which histology may be required. H&E-stained sections will reveal the laminated membrane of young hydatid cysts but it may not be possible to differentiate the early migratory stages of the different parasites as indicated by Lloyd *et al* (**)."

Lines 344 to 349: The paragraph should be replaced by the following: "Early migrating parasites can cause reddish haemorrhagic and later grey purulent tracks in the brain and in heavy infections the sheep may have a meningoencephalitis. Clinical signs due to the mature cyst relate to pressure atrophy of adjacent nervous tissue and vary according to location in the brain. There may be impaired vision or locomotion if cysts are in the cerebral hemisheres and the sheep gradually may be unable to feed and will become emaciated. Cerebellar cysts may precipitate more acute and severe signs of ataxia or opisthotonus. In heavy infections, parasites migrate and begin development in other tissues, but they die early. These produce small lesions, 1-2 mm or so in size, that first contain an encapsulated cyst, then eosinophilic, caseous material that later may calcify."

Line 360: Add the words "compared with those detected following dissection and slicing," after "were detected".

Line 366: At the end of the paragraph, add the following sentences: "Wanzala *et al* (***), also in Kenya, described meat inspection insensitivity detecting cysticerci in only 50% of naturally or artificially infested cattle. Their observations indicated that a number of viable cysticerci may be missed."

Line 424: Add the following sentence "The S3Pvac *T.solium* subunit vaccine has indicated field efficacy against natural infection but requires further development (*****)" after "*T. solium* (4)."

Add the following references:

* KHALIL, L.F., JONES A. & BRAY R.A. (1994). Keys to the cestode parasites of vertebrates. Wallingford, Oxon, UK: CAB International.

** LLOYD S., MARTIN S.C., WALTERS T.M.H. & SOULSBY E.J.L. (1991) Use of sentinel lambs for early monitoring of the South Powys Hydatidosis Control Scheme: prevalence of *Echinococcus granulosus* and some other helminths. Veterinary Record 129, 73-76

*** WANZALA W., ONYANGO-ABUJE J.A., KANG'ETHE E.K., ZESSIN K.H., KYULE N.M., BAUMANN M.P., OCHANDA H. & HARRISON L.J. (2003) Control of *Taenia saginata* by post-mortem examination of carcasses. Afr Health Sci. 3(2), 68-76

**** Lightowlers M.W. COLEBROOK A.L., Gauci C.G. , GAUCI S.M., KYNGDON C.T., MONKHOUSE C., VALLEJO RODRIQUEZ C., READ A.J., ROLFE R.A. & SATO C. (2003). Vaccination against cestode parasites: anti-helminth vaccines that work and why. *Veterinary Parasitology,* 115, 83-123

***** SCIUTTO E., ROSAS G., HERNANDEZ M., MORALES J., CRUZ-REVILLA C. TOLEDO A., MANOUTCHARIAN K., GEVORKIAN G., BLANCAS A., ACERO G., HERNANDEZ B., CERVANTES J., BOBES R.J., GOLDBAUM F.A., HUERTA M., DIAZ-OREA A., FLEURY A., de ALUJA A.S., CABRERA-PONCE J.L., HERRERA-ESTRELLA L., FRAGOSO G. & LARRALDE C. (2007) Improvement of the synthetic tri-peptide vaccine (S3Pvac) against porcine *Taenia solium* cysticercosis in search of a more effective, inexpensive and manageable vaccine. Vaccine, 25, 1368-1378

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.10.8. Cryptosporidiosis

Country making the comments: European Community

Date

General Comments

This is an excellent description of the state of the art. It has been revised by a group of specialists.

When used alone, the word "cryptosporidium" should be neither capitalised nor italicised.

Specific Comments (add continuation sheets if required)

Line 5:

- "16 ‘valid’ species"; there is a contradiction to line 63: "15 ‘valid’ species"; table 1 lists 16 species.

- "C galli" should be added after "meleagridis".

Line 16: C. galli should be mentioned at this point.

Line 29: The proposed modification should be rewritten as such: "Those typing and subtyping systems used for veterinary *(*and human*)* samples should also be used for environmental samples, to avoid any confusion arising from the use of different systems during the investigationthe- of disease outbreaks with both veterinary and public health implications. However the subtyping systems will need further research to provide methods sensitive enough to be able to use on low level positive environmental samples. Further problems of subtyping systems is that they are not yet highly discrimatory for the majority of cryptosporidium species."

Line 63: see above Line 5.

Line 75: The sentence "*Cryptosporidium bovis* is a highly prevalent species that infects primarily post-weaned calves (9)" should be deplaced to line 72, after "(Table1)."

Line 80: The following sentence should be added at the end of this paragraph: "Some may become recognised as species as further research is carried out."

Table 1: Oocyst dimensions: some figures are a bit meticulous (e.g. 5.05 x 4.41 µm). It might be better to provide a measure of uncertainty or the range of measurements for length and width, as done for *C. andersoni*.

Line 630: Where can positive and negative slides or reference samples be obtained from?

Line 704 ff: It would be good to have not only data on the analytical, but also on the diagnostic sensitivity,

Line 709: Shouldn't ‘that’ read ‘than’?

We also would like to precise some data concerning Cryptosporidium galli.

1. Whereas C. galli was described at the first time in the Department of Pathology and Parazitology of the State Veterinary Institute in Prague by I. Pavlásek in dead adult hen in the year 1999 (not in chicken as it is mentioned by mistake in the text – line number 67). Subsequently C.galli was described by the same author in some wild and exotic birds (as a new birds type of cryptosporidium as it was confirmed by genotyping of oocysta in Australia).

We recommend to add in the Chapter A - Introduction - line No 67 the following: C.galli in adult hens and some wild and exotic birds …with reference to (see below).

If in this line the finding of this type of cryptosporidium (C.galli) is mentioned as the finding in finches, this information is not correct.

2. In connection with the above mentioned it is necessary to amend the text into the line No 139: Cryptosporidium galli is a disease of adult hens and some wild and exotic birds.

3. In line No 154 – Cryptosporidium parvum, not Cryptosporidium Parvum.

4. In the list of References to add the list of publications about the first findings and description of endogen and morfometric parameters of oocyst of C. galli:

PAVLASEK,I. (1999).Kryptosporidie: biologie, diagnostika, hostitelské spektrum, specificita a vztah k životnímu prostředí (Cryptosporidia: biology, diagnosis, host spectrum, specificity and the environment). Remedia Klin Microbiol, 3(9):290-301, In Czech

PAVLASEK,I. (2001). Nálezy kryptosporidií ve žlaznatém žaludku u slepic a u volně žijících a exotických ptáků odchycených z volné přírody (Findings of cryptosporidia in the proventriculum of hens and in wild and exotic birds). Veterinářství, 3:103-108, In Czech

ADVICE FOR MEMBER COUNTRY COMMENTS

Chapter Number and Title: 2.10.12. Verocytotoxogenic *Escherichia coli*

Country making the comments

Date

General Comments

The chapter covers very well all the relevant aspects related with the diagnostics of VTEC.

This chapter is well written and is clearly laid out to ensure understanding. In recent years there has been an increase in the reporting of non O157 VTEC so an increase in the discussion of the occurrence and distribution of these organisms would be welcome.

A mention and reference to subtyping of Intimin and VTs would also be helpful, as would an indication of the use of multiplex and real time PCR as well as microarrays for such subtyping.

A mention of diagnostic laboratories that can provide a flagellar typing and phage typing for VTECs would be useful.

Specific Comments (add continuation sheets if required)

Summary, line 16-17: The indication of so many animal species without discriminating among them might be misleading for the reader. Suggestion: Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from pigs, cats, dogs, chickens and wild birds.

Introduction, line 68-69: See the above comment. Suggestion: Ruminants represent the main natural host of VTEC and are generally healthy carriers of the organisms. VTEC have also been isolated from the faeces pigs, cats, dogs, chickens and wild birds, which can be transiently colonized by the organisms.

Lines

16 Replace 'still' with 'may be associated'.

24 Insert 'contaminated' before 'water'.

41 Insert 'based' after 'nucleic acid'.

61 Replace 'risen' with 'increase'.

68 Insert 'carriers' after 'healthy'.

69 Insert ‘normally’ before ‘represent’.

71 Replace ‘by’ with ‘via’.

72 Replace infected with ‘contaminated’ and refer also to contaminated irrigation and wash water for vegetables.

74 Sheep and goats are also important reservoirs.

94 Refer also to recto-anal junction swabs and ‘rope’ samples.

95-99 This section is out of place in ‘Samples’.

103 Refer also to ‘Supershedders’, environmental recycling, opportunities for pooling samples, use of boot swabs.

116 Both direct plating after dilution and enrichment-IMS combined best.

117 BPW without antibiotics best overall for all samples.

124 24h incubation increases loss through overgrowth so would need 6h subculture as well.

130 IMS beads mixed with aliquot of incubated broth, not the sample - also refer to circulating IMS recovery systems.

141 Most O157:H- not actually non-motile - just lacking full expression of H7 antigen. Not all H7- strains are sorbitol fermenters - reword. Increased discussion of identification of sorbitol fermenters would be useful.

157 A bit more detail of alternative media and advantages as well as dilutions, spiral plating and potential for acid enrichment would be useful.

168 Replace ‘but’ with ‘and’.

169 Delete ‘for research purposes only’.

172 Is RMAC still thought to be reliable?

215 Insert ‘most’ after ‘*E.coli*’.

221 Refer to GadA PCR and other PCR confirmatory tests.

235 Insert ‘normally’ after ‘will’.

236 Insert ‘the’ before ‘sensitivity’.

238 Replace ‘all’ with ‘most’.

241 ‘genes’ in non-italics.

246 Refer to antimicrobial resistance being uncommon in VTEC strains in most countries.

257 Insert ‘molecular’ before approaches. Briefly describe the use of Intimin and VT subtyping and significance in epidemiology and source attribution.

263 Delete ‘the’ before food.

269 Insert comma after ‘isolation’.

286 Refer to the fact that availability of commercial diagnostic tests is subject to rapid change so Internet searches and consulting Reference Laboratory advised when latest information is required.

316 Insert ‘in most countries’ after ‘concern’.

325 Is this 2h time limit validated? - for robust organisms some delay may reduce competitors.

326 -70° C for long-term storage only - not prior to testing if >24h delay.

327 Greater dilutions may also be beneficial.

328 Elevated temperature may reduce excessive competitors in some cases.

331 Is Dynal the only supplier of labelled immunomagnetic particles for VTEC O157?

346 10µl loop better for spreading a well - also is the suggested streak pattern really the best for reducing competitors and providing individual colonies compared with 4-quadrant streaking?

356 Can colonies be pooled, or a sweep tested as initial screen?

363-371 What is sensitivity and specificity of ONPG and Indole screening?

372 Replace ‘strips’ with ‘kits’.

439 Clarify whether the first sentence relates to human infection.

CHAPTER 2.3.13.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB) in 1986. It is a transmissible spongiform encephalopathy or prion disease. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 2.4.8. Scrapie).

The epizootic of BSE can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat-and-bone meal included in proprietary concentrates or feed supplements. Initial cases of BSE in some countries were considered to be the result of exports from GB of infected cattle or contaminated meat-and-bone meal, although exportations from other countries are now implicated. In others, initial cases are clearly indigenous, with no clear link with imported meat-and-bone meal, suggesting that earlier, undetected, cases may have occurred. As a result of control measures, the epizootics in many countries are in decline. Cases of BSE currently occur throughout most of Europe and have been detected in Asia and North America.

Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposures to brain tissue from affected cattle. The BSE agent is also believed to be the common source, via dietary routes, of transmissible spongiform encephalopathies (TSEs) in several other species of ruminants and in species of felidae. There is evidence of a causal link between the BSE agent and the variant form of the human TSE, Creutzfeldt-Jakob disease (vCJD). Recommendations for safety precautions for handling BSE-infected material now assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: *In GB, BSE had a peak incidence in cattle aged between 4 and 5 years. The clinical course is variable but can extend to several months. Overt clinical signs are sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are eliminated. Early clinical signs may be subtle and mostly behavioural, and may lead to disposal of affected animals before suspicion of BSE is triggered. In countries with a statutory policy toward the disease, clinically suspect cases must be slaughtered, the brain examined and the carcass destroyed. Now, in most countries, active surveillance identifies infected cattle before, or without, the recognition of clinical signs. No diagnostic test for the BSE agent in the live animal is presently available. The nature of the agents causing the TSE is unclear. A disease-specific partially protease-resistant, misfolded isoform of a membrane protein PrP^c, originally designated PrP^{Sc}, has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the principal or sole component of the infectious agent. Confirmation of the diagnosis, formerly by histopathological examination of the brain, is now, therefore, by the application of immunohistochemical (IHC) and/or immunochemical methods to brain tissue for the detection of PrP^{Sc}. PrP^{Sc} can be detected in specific neuroanatomical loci in the CNS of affected cattle by immunohistochemical methods in formalin-fixed material, or by immunoblotting and other enzyme immunoassay methods using unfixed brain extracts.*

Transmission from infected brain tissue, usually to conventional or transgenic mice, is the only practical method currently available for detection of infectivity and has an important role in the confirmation or characterisation of agent strains. Claims that variant forms of BSE have been detected remain to be ~~substantiated~~further elaborated by full characterisation of isolates. They have arisen solely from active surveillance where some variations in immunochemical detection patterns have prompted claims of strain differences. In the majority of instances the claim is based ~~solely~~ mostly on western immunoblot banding pattern, but proof of transmissibility to rodents has now been demonstrated, and transmission studies to cattle are in progress.

48 ***Serological tests:*** *Specific immune responses have not been detected in TSEs.*

49 ***Requirements for vaccines and diagnostic biologicals:*** *There are no biological products*
50 *available currently. Commercial diagnostic kits for BSE are available and are used for diagnosis of*
51 *BSE in many countries.*

52 ## A. INTRODUCTION

53 BSE is a fatal disease of domestic cattle, cases of which were first recognised in Great Britain (GB) in November
54 1986 (23, 33). It is a transmissible spongiform encephalopathy (TSE) or prion disease, originally typified in animal
55 species by scrapie of sheep. Prion diseases are defined by the pathological accumulation, principally and
56 consistently in the central nervous system (CNS) and more variably in the lymphoreticular system (LRS), of a
57 misfolded, partially protease-resistant, isoform of a highly conserved, host-encoded membrane protein (PrP^C),
58 which was originally designated PrP^{Sc}. The function of PrP^C remains unclear. PrP^{Sc} is the only disease-specific
59 macromolecule identified in the scrapie-like diseases. It is also variably referred to as PrP^{res}, to denote the
60 proteinase resistant property of the pathological protein, PrP^d for disease-specific and PrP^{bse} specifically in BSE.
61 Here PrP^{Sc} is used generically to refer to the abnormal isoform of PrP^C, but for accuracy the term PrP^{res} is
62 adopted when referring to the extracted proteinase-resistant form of the protein. The favoured scientific view is
63 that the agent is composed entirely of the disease-specific isoform of PrP and that the altered form is capable of
64 inducing conversion of the normal form: the protein only or 'prion' hypothesis. Data in support of alternative
65 hypotheses, such as viral or bacterial origins or the involvement of cofactors such as mineral imbalances, remain
66 elusive. The molecular basis for strain variation is still unclear, but according to the prion hypothesis strain
67 characteristics are encoded in different conformations of the prion protein.

68 Characterisation of BSE isolates from GB by transmission to mice has shown that it is caused by a single major
69 strain of agent that differs from characterised strains of the scrapie agent in sheep (4). Uniformity of the pathology
70 among affected cattle has also supported the notion of a single BSE strain and enabled the definition of a
71 consistent disease phenotype for BSE (6, 26). This specific pattern of neuropathology in the host species is
72 important in the phenotypic characterisation and consequent case definition of BSE used for confirmation of the
73 disease. Reports since 2003 of variant features of pathology and/or molecular characteristics, arising solely from
74 the active surveillance programmes in several countries, other than GB, have raised issues of possible agent
75 | strain variations of prion disease in cattle (3, Buschmann et al., 2006; 7, 40) Whether or not such findings
76 represent true strain variation of the BSE agent, or different forms of prion infections of bovines, remains to be
77 proven. Because of their detection by active surveillance, none of the findings can be correlated with clinical
78 histories, and most focus only on western immunoblotting data (3, 40). The most comprehensive description,
79 providing immunohistochemical, histopathological and western immunoblotting characterisation relates to two
80 aged cows in Italy (7). Transmissibility of certain isolates to mice, with features distinct from previous BSE
81 | transmissions has been confirmed (2; Buschmann et al., 2006). Transmission studies of other isolates in cattle are
82 in progress. An interesting common feature is that most of these isolates originate from older cattle.

83 The initial epidemiological studies of BSE in GB established that its occurrence was in the form of an extended
84 common source epizootic, due to feed-borne infection with a scrapie-like agent in meat-and-bone meal used as a
85 dietary protein supplement (1, 35). Although recorded initially in the United Kingdom (UK), BSE has now occurred,
86 albeit at lower incidence, in many countries involving imported and/or indigenous cattle. Such cases are most
87 likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal from
88 countries with occurrences of BSE, including historically, the UK. It is clear that infection has subsequently been
89 propagated within countries in which cases have occurred as highlighted by the evaluation of Geographical BSE
90 Risk (GBR) in many countries by the Scientific Steering Committee of the European Union (11). Indeed, in some
91 countries, the only cases detected reflect indigenous exposure rather than direct linkage with imported
92 contaminated feed. Current statistics on BSE occurrence around the world are provided by the OIE (37).

93 There is no evidence of horizontal transmission of BSE between cattle and little data to support the existence of
94 maternal transmission. Epidemiological and transmission studies have not revealed evidence of a risk from semen
95 or milk or through embryos.

96 As a result of control measures, the epizootics in the UK and many other countries have declined, or show the
97 effects of controls in the form of changes in age-specific incidence. In some countries the controls have not been
98 in place long enough for the effects to be recognised. Interpretation of the status of epizootics has been enhanced
99 by the introduction of active surveillance using rapid diagnostic tests, which have detected infected animals that
100 have not been recognised as clinically suspect cases. While such active surveillance is capable of detecting a
101 proportion of preclinical cases, retrospective investigation at farms of origin frequently confirms that some signs
102 have been presented before slaughter, but had not triggered consideration of a clinical diagnosis of BSE.

103 The novel occurrence of TSEs in several species of captive exotic bovidae and felidae and in domestic cats
104 during the course of the BSE epizootic is attributed to and, for several affected species, shown, to have been
105 caused by the BSE agent (20). Exposure is presumed to have been dietary.

106 The emergence of a new form of the human prion disorder Creutzfeldt-Jakob disease (CJD), termed variant CJD
107 (vCJD) in the UK (36) has also been shown by transmission and molecular studies (5, 8) to be causally linked to
108 the BSE agent. Dietary exposure is considered the route of infection. In the past, no connection has been
109 established between the exposure of humans to agents causing animal spongiform encephalopathies and the
110 occurrence of the human TSE and thus BSE presents a precedent as a zoonotic TSE. It is therefore now
111 recommended that safety precautions for handling the BSE agent be based on the assumption that BSE is
112 transmissible to humans. The epizootic of vCJD in the UK in individuals homozygous for MM at codon 129 of the
113 PrP gene, peaked in 2000; small numbers of cases have occurred in some other countries.

114 Consequent on the occurrence of vCJD, workers conducting necropsies on BSE-suspect animals or handling
115 tissues derived from such animals, must conduct the work under containment level 3, (see Chapter 1.1.6),
116 sometimes with derogations and the laboratory must comply with national biocontainment and biosafety
117 regulations to protect staff from exposure to the pathogen. Recommended decontamination procedures may not
118 be completely effective when dealing with high-titre material or when the agent is protected within dried organic
119 matter. Recommended physical inactivation is by porous load autoclaving at 134°C–138°C for 18 minutes at
120 30 lb/in^2. However, temperatures at the higher end of the range may be less effective than those at the lower end
121 and total inactivation may not be achieved under certain conditions, such as when the test material is in the form
122 of a macerate. Disinfection is carried out using sodium hypochlorite containing 2% available chlorine, or 2 N
123 sodium hydroxide, applied for more than 1 hour at 20°C for surfaces, or overnight for equipment (29).

124 # B. DIAGNOSTIC TECHNIQUES

125 ## 1. Identification of the agent

126 **Clinical BSE** occurs in adult cattle, and most cases have been observed in dairy cattle aged 4–5 years. Onset of
127 clinical signs is not associated with season or stage of breeding cycle. BSE has an insidious onset and usually a
128 slowly progressive course (21, 34). Occasionally, a case will present with acute signs and then deteriorate rapidly,
129 although frequency of observation is a significant factor in determining early clinical signs. Presenting signs,
130 though variable, usually include behavioural changes, apprehension, and hyper-reactivity. For example, affected
131 cows may be reluctant to enter the milking parlour or may kick vigorously during milking. In dry cows especially,
132 hind-limb incoordination and weakness can be the first clinical features to be noticed. Neurological signs
133 predominate throughout the clinical course and may include many aspects of altered mental status, abnormalities
134 of posture and movement, and aberrant sensation, but the most commonly reported nervous signs have been
135 apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. The intense pruritus characteristic of
136 some sheep with scrapie is not prominent in cattle with BSE, though in a proportion of cases there is rubbing and
137 scratching activity. Affected cows will sometimes stand with low head carriage, the neck extended and the ears
138 directed caudally. Abnormalities of gait include swaying of the pelvic quarters and pelvic limb hypermetria;
139 features that are most readily appreciated when cattle are observed at pasture. Gait ataxia may also involve the
140 forelimbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and
141 recumbency, can dominate the clinical picture. Reports of reduced rumination, also bradycardia and altered heart
142 rhythm, though not specific signs, suggest that autonomic disturbance is a feature of BSE. General clinical
143 features of loss of bodily condition, decreasing live weight, and reduction in milk yield often accompany nervous
144 signs as the disease progresses. There has been no change in the clinical picture of BSE over the course of the
145 epizootic in the UK (21, 34). Clinical signs are essentially similar in other countries where BSE has occurred. The
146 protracted clinical course, extending usually over a period of weeks or months, would eventually require slaughter
147 on welfare considerations. However, a statutory policy to determine the BSE status of a country requires
148 compulsory notification and diagnostic investigation of clinically suspect cases, their slaughter and post-mortem
149 examination of the brain. Early in the disease course, the signs may be subtle, variable and nonspecific, and thus
150 may prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases, together
151 with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders,
152 will establish the essential progression of signs. Some early clinical signs of BSE may show similarities with
153 features of nervous ketosis, hypomagnesaemia, encephalic listeriosis and other encephalitides. Subtle signs may
154 sometimes be exacerbated following stress, such as that caused by transport. Video clips of cattle affected by
155 BSE may be downloaded from the web site of the European Commission (EC) TSE Community Reference
156 Laboratory/Veterinary Laboratories Agency (VLA) (13). DVD or videotape footage of the clinical signs is available
157 from this and other sources (30).

158 **The laboratory diagnosis** of BSE has evolved in concert with increasing knowledge of the disease and technical
159 advances (15). In the absence of *in-vitro* methods for isolation of the causative agent, the historical basis of
160 confirmation of the diagnosis in this group of diseases was the demonstration of the morphological features of

spongiform encephalopathy by histopathological examination. This remains necessarily, by definition, the only method by which this characteristic vacuolar pathology can be diagnosed. The original diagnosis of BSE was based on the histopathological features of a scrapie-like spongiform encephalopathy and the electron microscopic visualisation of fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrP^{res}, in detergent extracts of affected brain. The material examined was invariably from suspect clinical cases. In GB, in the light of the rapidly increasing epizootic in the late 1980s, histopathological diagnosis based on examination of a single section of medulla oblongata taken at the level of the obex, was validated against more extensive examination of the brainstem (30). This simplified approach enabled modification of sampling of the fresh brain; instead of whole brain removal, the required section was taken from the brainstem removed via the *foramen magnum*, using customised instrumentation. With increasing recognition of the diagnostic specificity of PrP^{Sc} and, with availability of appropriate antibodies and increasing efficiency of detection methods, immunochemical methods of disease-specific PrP detection, including immunohistochemical (IHC) techniques and Western blotting/SAF-immunoblotting were used, in addition to histopathology, to confirm the diagnosis. The introduction of more rapidly performed *in-vitro* methods for the detection of PrP^{Sc} led to implementation of a variety of 'rapid', mostly enzyme-linked immunosorbent assay (ELISA)-based, tests, conducted on sub-samples of medulla oblongata, and these have become the principal approach for active surveillance diagnosis. Such tests provide a preliminary screening from which positive or inconclusive results are subject to examinations by immunohistochemical or Western blot confirmatory methods. Rapid test strategies are currently the main approach by which cases are detected, and enabling their wider use as part of the confirmatory process has been agreed in principle **[It is suggested that a ref to the OIE doc recently agreed by the Standards Commission could go here)]**

The use of a particular method will depend on the purpose to which the diagnosis is to be applied in the epidemiological context, and its validation for that purpose. This range of purposes will extend from confirmation of the clinical diagnosis in the control of epizootic disease to the screening of healthy populations for evidence of covert or preclinical disease. The case definition adopted will also differ according to whether the diagnostic method is to be applied for confirmation of a clinical case or for screening of a population. For the ~~former~~ final confirmation of any case, it is important to use approaches that can monitor the pathological determinants of the phenotype of BSE. ~~Care should be taken in the interpretation of data using methodologies that do not enable careful cross-referencing with the standards for confirmatory diagnosis that are defined here.~~~~Only approved methods shall be applied for the final confirmation or dismissal of a BSE suspect case.~~ Without appropriate comparison with previously published criteria defining the BSE phenotype and in the absence of transmission studies, diagnostic results that claim the identification of a new strain are ~~unjustified~~premature. Quality control (QC) and quality assessment (QA) are essential parts of the testing procedures and advice can be supplied by the OIE Reference Laboratories (13, 38) Whether BSE-infected animals are to be identified by passive or active surveillance, it is good practice to detect and confirm disease by a combination of at least two test methods. So, whether the primary test is one of the confirmatory methods described below, or a rapid test, it is important to apply a secondary test to confirm a positive or inconclusive primary test result . Where there is a conflict between primary and secondary test results, further tests using immunohistochemistry or SAF-immunoblot (or approved alternative) should be applied or samples should be submitted to an OIE Reference laboratory for resolution.

a) Sample preparation

The BSE status of a country, the relative implementation of passive and active surveillance programmes and the diagnostic methods applied, will all influence sampling strategy.

In all circumstances of passive surveillance of neurological disease in adult cattle where the occurrence of BSE within a country or state has not been established or is of low incidence, it is recommended that clinically suspect cases are subjected to a standard neuropathological approach in which representative areas of the whole brain are examined. Moreover, care must be taken to preserve ~~the~~ suitable fixed and fresh ~~tissue~~ brain samples for the immunohistochemical and immunochemical detection of PrPSc ~~in the fixed of frozen brain~~. ~~-~~Departure from this approach may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE. Cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution preceded, if necessary, by sedation. The brain should be removed as soon as possible after death by standard methods.

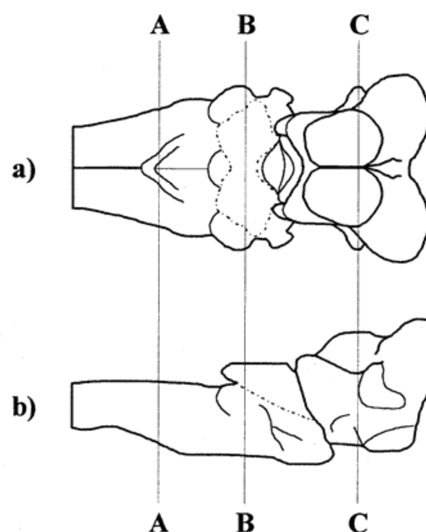
Histopathology and immunohistochemical examinations are carried out ~~In~~initially on ~~,~~ a single block (0,5 –1 cm in width) cut at the obex of the medulla oblongata (Fig. 1a and b, level A–A representing the centre of the block for examination) which should be selected for fixation ~~(~~for at least ~~1~~3-5 days in 4% formaldehyde solution (i.e. 10% formal saline or 10% neutral buffered formalin) and subsequent histological processing by conventional paraffin wax embedding methods for neural tissue if conventional fixation protocols are used~~)~~.

Fresh material for ~~potential~~ use in confirmatory immunoblotting ~~tests~~ to detect disease-specific PrP should be taken initially ~~ideally,~~ as a complete coronal section (2–4 g) from the medulla, immediately rostral, or caudal, to the obex block taken for fixation. Alternatively, the medulla at the level of the obex could be hemisectioned, as described for active surveillance (see below).

220

221 All other brain areas should be subdivided by a saggital cut paramedian (0.5-1 cm off the median) cut into
222 two halves.

223 The smaller half-portion is reserved for the Tissue for PrP^{res} detection by immunochemical methods (e.g.
224 SAF-immunoblot) and is stored frozen prior to testing (if testing is not done immediately after sampling).
225 After sampling of obex region for fixation and sampling of fresh tissue, Tthe smallerlarger half-portion of the
226 remaining brain tissue is placed, intact, in approximately 4–6 litres of 4% formaldehyde 10% formol saline
227 formaldehyde fixative (see above), which should be changed twice weekly-. After fixation for 2 weeks, the
228 brain is cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem (detached
229 from the rest of the brain) into smaller coronal pieces, similarly to the initial removal of the obex region, but
230 leaving intact the remaining diagnostically important cross-sectional areas at the levels of the the cerebellar
231 peduncles and the rostral colliculi (Figure 1a and b, levels B–B and C–C, respectively). Depending on some
232 other factors (temperature, agitation, thickness of tissue block, use of microwave etc) the fixation time for
233 these small pieces of brainstem may be reduced to 21-2–5 days **[however , evaluation of the effects of**
234 **this kind of processing on subsequent IHC protocols needs to satisfy proficiency testing standards]**
235 The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard
236 two weeks' fixation.



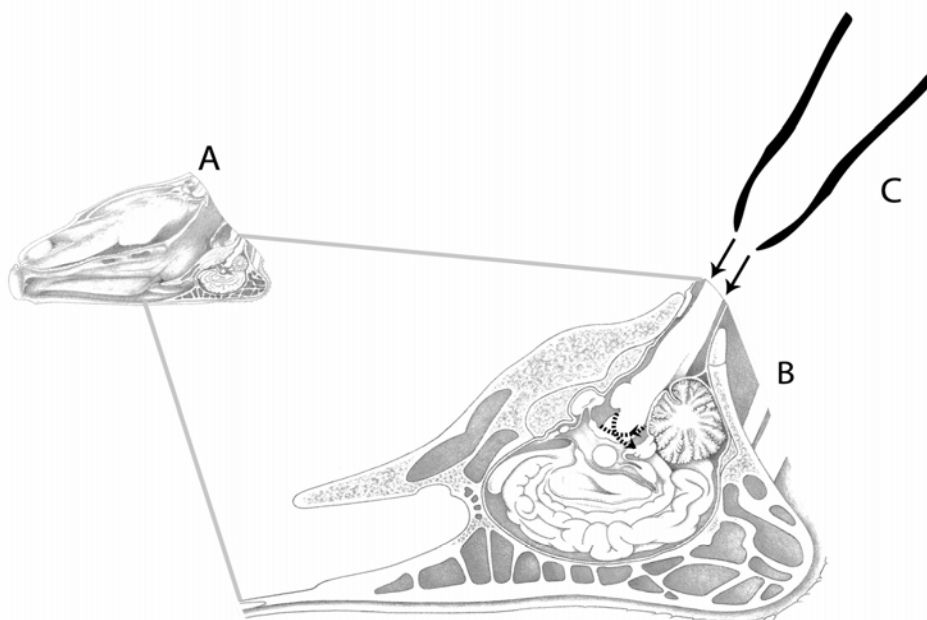
237 ***Fig 1**. Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects. f*
238 *Recommended levels at which sections should be taken:*
239 *A–A = medulla, at the obex; B–B = medulla through caudal cerebellar peduncles;*
240 *C–C = midbrain through rostral colliculi.*

241 **When the occurrence of BSE in a particular country has been established in the indigenous cattle**
242 **population**, and there is evidence that the distribution of lesions and other phenotypic determinants of the
243 known cases, are consistent with that seen in the brains of cattle from the UK epizootic, it is adequate,
244 although not ideal, for monitoring purposes, to remove the brainstem alone.

245 This can be achieved via the *foramen magnum* without removal of the calvarium (Fig. 2). This will reduce the
246 amount of fixative required and the time and equipment required, thereby lowering costs and improving
247 safety, while maintaining representation of the major target areas for histological examination. This is readily
248 achieved for collecting large numbers of samples, either for passive surveillance, dealing with large numbers
249 of suspected cases, or for an active surveillance programme and can be achieved for the latter at abattoirs.
250 The brainstem is dissected through the *foramen magnum* without opening the skull by means of a specially
251 designed spoon-shaped instrument with sharp edges around the shallow bowl (Fig. 2). Such instruments are
252 available commercially, made of plastic or metal. It is possible that variations in technique, including
253 orientation, are required with different forms of the instrument, thus highlighting the need for training of
254 operators once there is agreement on equipment to be used. Under abattoir conditions it has also been
255 shown possible to obtain expulsion of intact brainstem via the *foramen magnum*, providing histologically
256 good material, by application of fluid pressure (air or water) (18) through the entry wound in the skull when

257 penetrative stunning has been used in slaughtering. Clearly the feasibility and efficacy of this method will be
258 dependent on the slaughter method and before implementation for routine use requires to be subjected to
259 risk assessment.

260 **Where the index case is identified through active surveillance**, the necessary brain areas for full
261 phenotypic characterisation are unlikely to be available. In most countries, brainstem alone is collected, even
262 before the first confirmation of BSE. Ideally, provision should be made for heads that have been sampled in
263 the course of active surveillance to be retained until the outcome of initial testing is available. This would
264 enable much more comprehensive sampling of the brain of positive animals and enable the recommended
265 approach to the characterisation of cases. This is particularly important if un-validated tests are used, and
266 where in the absence of direct comparison with the methods described here results in claims that new
267 phenotypes have been identified. Where rapid immunoassays are used as the primary surveillance tool, in
268 the absence of a diagnosis of BSE having ever been made in a country, a modified approach may be
269 necessary to make provision for a further pathological morphological and immunohistochemical examination
270 that would allow identification of disease phenotype.



271

272 **Fig. 2.** *After the head has been removed from the body by cutting between the atlas vertebra and the occipital*
273 *condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the*
274 *brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing of cranium).*
275 *The instrument (C) is inserted through the foramen magnum (⟵) between the dura mater and*
276 *the ventral/dorsal aspect (depending upon the specific approach) of the medulla and advanced rostrally,*
277 *keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a*
278 *side-to-side rotational action. This severs the cranial nerve roots without damaging the brain tissue. The*
279 *instrument is passed rostrally for approximately 7 cm in this way and then angled sharply (i.e. toward the*
280 *dorsal/ventral aspect of the brainstem, depending on the approach [......▸]), to cut and separate the*
281 *brainstem (with some fragments of cerebellum) from the rest of the brain. The instrument, kept in the*
282 *angled position, is then withdrawn from the skull to eject the tissue through the foramen magnum.*

283 • **Sampling of brainstem in active surveillance with use of rapid tests**

284 The sampling and processing of the brain tissue for use in the rapid test mustshouldshould be carried out
285 precisely as specified by the supplier or manufacturer of the test method or kit.**[CRL comment – this**
286 **assumes that all kit manufacturers had their sampling method specifications approved initially,**
287 **which is not the case]**. The preferred sample for immunoassay should be at, or within 1.0 cm rostral, or
288 caudal to, the obex, based on the caudo-rostral extent of the key target sites (Fig. 3) for demonstration of
289 PrP^{Sc} accumulations and the evaluation of sampling for rapid tests. The choice of target site has to take into

account the subsequent method of confirmation. At least a hemisection of the medulla at the level of the obex should be fixed for immunohistochemistry/histology. ~~For example, the inability to examine medulla at the obex histologically may prevent the detection of bilateral vacuolation.~~ Sampling the medulla rostral or caudal to the obex for rapid testing does not compromise examination by histological or immunohistochemical means. However, to obtain comparable samples for rapid *and* confirmatory testing, sampling by hemisection of the medulla at the level of the obex is preferable. ~~Sampling the medulla rostral or caudal to the obex, for rapid test does not compromise examination by~~ While there is resultant loss of the ability to assess the symmetry of vacuolar changes, this approach is less likely to compromise the more important immunohistochemical examination. If hemisectioning is adopted however, it becomes critical to ensure that the target sites are not compromised in either sample. For example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (target areas for lesions in cattle with BSE) are small, and lie relatively close to the midline (Fig. 3) If sampled tissue is autolysed to the point where such anatomical orientation is not possible, an unidentified aliquot can still be taken and tested. A positive result in such cases is still a valid result, but a negative test result cannot be taken to indicate a negative animal, and it should be interpreted with caution and reported with appropriate qualification. ~~and caution~~

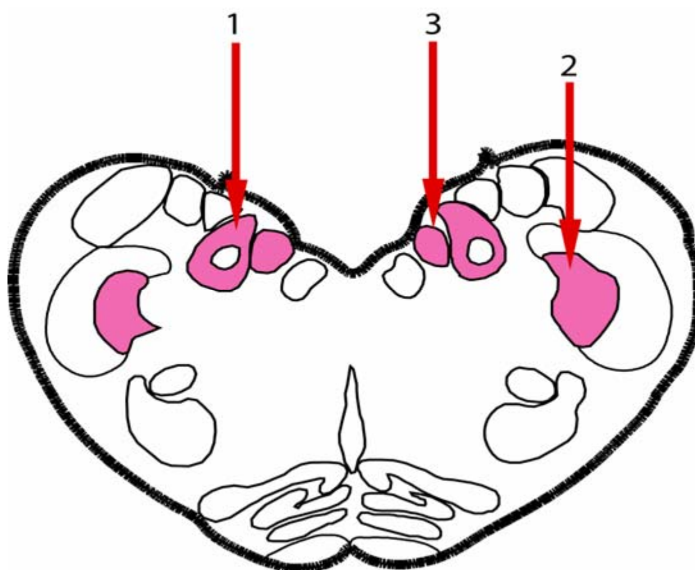


Fig 3. *Cross section of the bovine brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE. These are principally the nucleus of the solitary tract* [*1*] *and the nucleus of the spinal tract of the trigeminal nerve* [*2*]*; but also the dorsal motor nucleus of the vagus nerve* [*3*]*. It follows that material taken for application of a rapid test must also include representation of these areas.*

Inaccurate hemisectioning could easily result in the complete loss of a target area for confirmatory testing, and significantly reduce the effectiveness of the surveillance programme. Failure to accurately sample target areas may also arise through inappropriate placement of proprietary sampling tools. Such approaches therefore need to be implemented with a very clear policy and monitoring programme for training and quality assurance of sampling procedures. Because of the specifically targeted distribution of PrP^{res}, sample size and location should be as detailed in the diagnostic kit or, if not specified, at least 0.5 g, ~~taken from t~~The diagnostic target areas for all confirmatory tests are detailed in Fig 3. Performance characteristics of some~~all~~ of the tests ~~(histopathology!) are~~ ~~may~~ may be compromised by autolysis, particularly due to loss of the ability to ensure inclusion of target areas in the sample.

b) ~~b)~~ Diagnostic examination

Historically the histopathological examination of the brain has been the method of choice for the diagnosis of transmissible spongiform encephalopathies. The development of highly sensitive immunobiochemical and immunohistochemical techniques for the detection of pathological PrP in the brain has meanwhile added many new diagnostic possibilities. Even more, the use of such techniques revealed that the histopathological examination misses up to 10% of the clinical BSE cases. Therefore immunobiochemical and immunohistochemical PrP^{Sc} detection techniques have nowadays become the standard methods for the active and passive TSE surveillance as well as for as confirmatory testing

330

331 **i)Histological examination**

332 Histopathology is no longer the diagnostic method of choice for investigation of suspect animals, or screening of
333 healthy populations. However, an awareness of the histopathological lesions is important, to facilitate detection of
334 cases when conducting histological examinations of non-BSE suspect cattle brains for differential diagnosis.
335 Sections of medulla–obex are cut at 5 µm thickness and stained with haematoxylin and eosin (H&E). The
336 histopathological examination of H&E sections allows confirmation of the characteristic neuropathological changes
337 of BSE (26, 32) by which the disease was first detected as a spongiform encephalopathy. These changes
338 comprise mainly spongiform change and neuronal vacuolation and are closely similar to those of all other animal
339 TSEs, but in BSE the high frequency of occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of
340 the medulla oblongata at the level of the obex, provides a satisfactory means of establishing a histopathological
341 diagnosis on a single section of the medulla (30) in clinical suspect cases. As in other species, vacuolar changes
342 in the brains of cattle, particularly vacuoles within neuronal perikarya, of the red and oculomotor nuclei of the
343 midbrain are an incidental finding (16). The histopathological diagnosis of BSE must therefore not rely on the
344 presence of vacuolated neurons alone, particularly in these anatomical locations.

345 A number of cases have been observed during the last years where no TSE-specific histopathological
346 abnormalities were detected, although these cases were demonstrated to be clearly positive by
347 immunohistochemistry. Therefore, a negative histopathological result must in any case be confirmed by
348 another confirmatory method (immunohistochemistry or Western Blot). Due to this observation, laboratories
349 can directly perform one of these methods without histopathology.

350 The diagnosis may be confirmed if completely typical morphological changes are present in the medulla at
351 the level of the obex, but, irrespective of the histopathological diagnosis, immunohistochemistry is now
352 routinely employed in addition, as unpublished evidence suggests that as many as 5% of clinical suspects
353 (which are negative on H&E section examination for vacuolar changes at the obex) can be diagnosed by
354 immunohistochemical examination. Clearly, this protocol, confined to examination of the medulla–obex, does
355 not allow a full neuropathological examination for differential diagnoses to be established, nor does it allow a
356 comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended that
357 whole brains are removed from all clinical suspects.

358 **ii) ~~c)~~ Detection of disease-specific forms of PrP**

359 The ~~universal~~ use of PrP detection methods now provide a disease specific means of diagnosis independent
360 of the morphological changes defined by the histopathological approach. Many laboratories have therefore
361 now supplemented or replaced histopathological examination by IHC and other PrP-detection methods. The
362 detection of accumulations of PrP^{res} is the approach of choice for surveillance programmes and for
363 confirmatory diagnosis. It is possible (but not desirable) to undertake immunohistochemistry for PrP on
364 material that has been frozen prior to fixation (10). Freezing prior to fixation will not compromise the
365 immunoreactivity of a sample, but it may compromise the identification of target sites that need to be
366 checked before a negative result can be recorded.

367 • **Immunohistochemical methods**

368 The IHC examination to detect PrP^{res} accumulation is applied to sections cut from the same formalin-fixed,
369 paraffin-embedded material of medulla at the level of the obex, as that used for the histopathological
370 diagnosis (32). Several protocols have been applied successfully to the IHC detection of PrP for the
371 diagnosis of BSE and although harmonisation toward a fully validated standardised routine diagnostic IHC
372 method would seem desirable, experience has indicated that it is much more important to recognise robust
373 methods that achieve a standardised output, as monitored by participation in proficiency testing exercises,
374 and by comparison with the results of a standardised model method in a Reference Laboratory. The
375 technique does not necessarily require lengthy tissue fixation, although for accuracy the guidelines
376 established for histopathology still apply and, providing the tissue can be adequately processed
377 histologically, it works well in autolysed tissues in which morphological evaluation is no longer possible (9,
378 22). However, it is still necessary to be able to recognise the anatomy of the sample to determine whether or
379 not target areas are represented. This is essential for a negative diagnosis, and may also be pivotal in
380 accurately interpreting equivocal immunolabelling, IHC detection of PrP^{res} accumulations approximates to
381 the sensitivity of the Western blotting method for detection of PrP^{res} (24). In combination with good
382 histological preparations, immunohistochemistry allows detection of PrP^{res} accumulations and, as this, like
383 the vacuolar pathology, exhibits a typical distribution pattern and appearance, it provides simultaneous
384 evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by
385 reference to the OIE Reference Laboratories (13, 38).

386

387 • **Western blot methods (e.g. SAF-immunoblot)**

- ~~Detection of PrP^{res} by SAF-purification followed by i~~Immunoblotting techniques, ~~is~~ are carried out on fresh (unfixed) ~~or frozen brain or spinal cord material, however, preferably taken from a site located closely to the obex.~~ tissue, and can be applied successfully even when tissue is autolysed (17). The SAF-immunoblot (13) was the first such method for use in BSE diagnosis, ~~and has been used for more than 20 years~~. and ~~I~~it has similar diagnostic sensitivity to the immunohistochemical techniques, and remains the method of choice (besides immunohistochemistry) for the confirmation or dismissal of a BSE suspicion
- In the last decade alternative methods have been ~~worked out~~developed that are less time-consuming and less costly. Most of these techniques utilize a precipitation of PrP^{Sc} using phosphotungstic acid (PTA) or by other chemicals (27) ,and some are commercially available. ~~e.g. in form of the commercially available Westernblot test (Biorad TeSeE WB)~~.

While WB methodology is now in general use around the world, analytical sensitivity when used to detect PrP^{res} varies significantly between methods and laboratories. Where in-house methods are preferred to published methods for confirmatory purposes, it is important that they are evaluated as being fit for purpose in consultation with an OIE Reference Laboratory

~~Detection of PrP^{res} by SAF-purification followed by immunoblotting techniques, is carried out on fresh (unfixed) or frozen brain or spinal cord material. Improvements in purification methods for extracting PrP^{res} have contributed to increased sensitivity of this method. This methodology provides a sensitive method of confirming diagnosis following initial suspicion of disease using more recent rapid tests (13, 27). Immunoblotting methods are relatively robust when applied to autolysed material (17).~~

~~The sensitivity of the available Western Blot protocols can vary considerably, depending on the sample preparation and PrP^{Sc} purification ahead of the immunoblotting. Currently, the most sensitive Western Blot methods are the SAF-immunoblot (Ref), a PrP^{Sc} precipitation by phosphotungstic acid (PTA) prior to immunoblotting (Ref), or a commercial Western blot purchased by BioRad (TeSeE Western Blot). Performance of the Prionics Check Western Blot or any Western Blot of proteinase K digested samples without further PrP^{Sc} concentration must be considered less suitable for confirmatory purposes.~~

- **Rapid test~~s~~ methods**

Automated rapid Western-blot and ELISA techniques have been developed that allow screening of large numbers of brain samples and are commercially available. Such techniques can be performed ~~within~~ in a few hours.~~rapidly, are more sensitive than the histopathological evaluation but comparable with that of SAF-immunoblot and immunohistochemistry. The~~(see EC ~~has conducted~~ evaluations of rapid tests for the detection of BSE on known IHC positive and negative sample groups (12)).

While many countries accept EU approval as an indicator of test performance, others have established their own evaluation mechanisms, most notably the United States of America, Canada and Japan (12). The OIE now also has an approval process and protocols for such evaluations are posted on the OIE web site (39).

The relative sensitivities of rapid tests, immunohistochemistry and other confirmatory methods remains to be determined. Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE. Evaluations completed in the EU were restricted to a comparison of the examination of a sample of brains of cattle identified as suspect clinical animals with histopathological changes characteristic of BSE and a sample of brains of cattle from New Zealand that were unexposed to BSE and histopathologically negative. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period, for example in surveys of post-mortem material collected from routinely slaughtered cattle. In countries conducting surveillance for the detection of the novel occurrence of BSE and in those countries in which a means, independent of the system of notification of suspect cases, of assessing the prevalence of BSE is considered necessary, these screening tests offer an efficient approach. Since their introduction for active screening in Europe from January 2001, such tests have been responsible for the identification of the majority of BSE-infected animals. In some countries, given the speed with which results can be obtained, the rapid tests are the preferred primary test, but confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by histopathology and/or immunohistochemistry or the application of an appropriate Western Blot protocol. Nevertheless, in 2006, the OIE accepted that through their use in active surveillance programmes, commercial rapid tests have proved themselves to be very effective, and consistent, provided they are performed by appropriately trained personnel. Indeed, at times they may out-perform the acknowledged standard of comparison if training and experience in the latter are deficient. Under such circumstances, it is now considered acceptable, even if not ideal, for rapid tests to be used in combination for both primary screening in active surveillance programmes and subsequent confirmation. It will be essential however to ensure that the choice of primary and secondary

test are compatible, and do not present a danger of generating false positive results for the same reason, if reagents are shared. Consequently, an algorithm of preferred test combinations will be maintained on the VLA web site to assist those who wish to resort to this approach instead of histopathology and immunohistochemistry, or SAF immunoblot for confirmation (5). The ideal combinations should include an ELISA and western blot method as they generate useful complementary data to assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue.

Under certain circumstances, an EU or OIE approved rapid test could be used for the confirmation of BSE in bovines following an initial reactive result with an approved rapid test. Such approval ~~would be~~ is dependent on a review of reagents used in each rapid test to ensure that the pairs of tests used ~~were~~ are compatible. On the basis of confidential data released by test manufacturers, a procedure is now available, and can be found at – reference here? and is summarised below

1. The confirmation must always be carried out in a National Reference Laboratory (NRL) for TSE.
2. The second test must include a negative control and a bovine BSE sample as positive control.
3. The second test must be a different test (in other words, two positive results involving the same test is insufficient for confirmation).
4.If a rapid Western blot is used as first test, this result must be documented and submitted to the NRL. ~~)~~
5. One of the two methods must be a Western blot.

The combination of the two rapid tests can only be used for the confirmation of a BSE case. A negative result by secondary test is insufficient to define a case as negative following a primary positive result. BSE suspect cases with discordant rapid test results must therefore be investigated further using either the SAF-immunoblot (or approved alternative) or IHC for the demonstration of abnormal PrP^{Sc}, or if these methods are not available, by histopathology. If histopathology is unable to confirm the initial reactive result, samples should be submitted to an OIE Reference laboratory for further examination

Although the test evaluation programmes conducted in Europe were in support of legislation on surveillance for BSE, the consequences are of relevance to other countries as well. The consequences of false-positive or false-negative results are so great that the introduction of new tests should be supported by thorough evaluation of test performance. Claims by test manufacturers should always be supported by data, ideally evaluated independently. It must be stressed that the process of full validation of all of these diagnostic methods for BSE has been restrained by the lack of a true gold standard and the consequent need to apply standards of comparison based on relatively small studies. There is therefore a continuing need for the publication of larger scale studies of assay performance, and none of the data published so far equate with recognised procedures for test validation for other diseases.

d) Other diagnostic tests

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.4.8 Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue (28) has been used as an additional diagnostic method for BSE and has been particularly useful when histopathological approaches were precluded by the occurrence of post-mortem decomposition. With modification, the method may be applied successfully to formalin fixed tissue. Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE, but does not offer the specificity or sensitivity available from IHC or immunoblotting methods. BSE infectivity can be shown by intracerebral/intraperitoneal inoculation or by feeding of mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. Further development of transgenic mice, such as those over-expressing the bovine PrP gene, may potentially offer bioassays with reduced incubation periods for BSE, but none as yet represent practical diagnostic tools.

There remains the need for a test for BSE that can be applied to the live animal and has a sensitivity capable of detecting PrP^{res} at the low levels, that may occur early in the incubation of the disease. As yet, the effectiveness of potential approaches has not been shown. The EC remains committed to the evaluation of *in-vivo* tests, and sets out protocols for the evaluation of such tests (14). The detection of certain protein markers of neurodegeneration, including apolipoprotein E (Apo E), the 14-3-3 protein and S-100 proteins in cerebrospinal fluid have not proved useful for diagnosis of preclinical cases of BSE. The diagnostic potential of the observation of IgG light chains as a surrogate marker for prion infection in the urine of scrapie infected hamsters (19, 25), has not been investigated for the diagnosis of BSE.

2. Serological tests

The infectious agents of prion diseases cannot easily be grown *in vitro* and do not induce a significant immune response in the host.

505 ## C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

506 There are no biological products available currently. As discussed previously, diagnostic kits have been licensed
507 for use in many countries.

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611 **NB:** There are OIE Reference Laboratories for Bovine spongiform encephalopathy (see Table in Part 3 of this
612 *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
613 http://www.oie.int/eng/oie/organisation/en_listeLR.htm#B115

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