TSE EURL

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SAMPLE REQUIREMENTS FOR TSE TESTING AND CONFIRMATION – EURL GUIDANCE.

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1. BACKGROUND

The first stage of all the current TSE diagnostic or screening tests involves the sampling of the central nervous system at the level of the brainstem, and the subsequent examination of the sampled tissue for the presence of disease- specific PrP using immunochemical methods.

As new, atypical, forms of disease have been identified in cattle (H-BSE and L-BSE), sheep and goats (atypical scrapie) it is becoming apparent that the cerebellum is also a key area for robust confirmation and classification of these variants.

PrP has proved to be the most consistent marker for all known forms of TSE, being present in the Central Nervous System (CNS) of all recognised clinically suspect TSE cases, and it has been shown experimentally that demonstrable accumulations of PrP arise in the CNS (and in a more variable way the lymphoreticular system) in advance of any clinical disease. It is thus a useful marker in pre-clinical animals, as well as in those presenting with overt disease.

The brain consists of multiple interrelated but anatomically and functionally distinct areas, and disease related PrP accumulation shows distinct anatomically-specific trophisms which result in clear-cut patterns of PrP accumulation (Figure 1). These patterns are specific both in end-stage disease, and through the pathogenesis of each form of TSE.

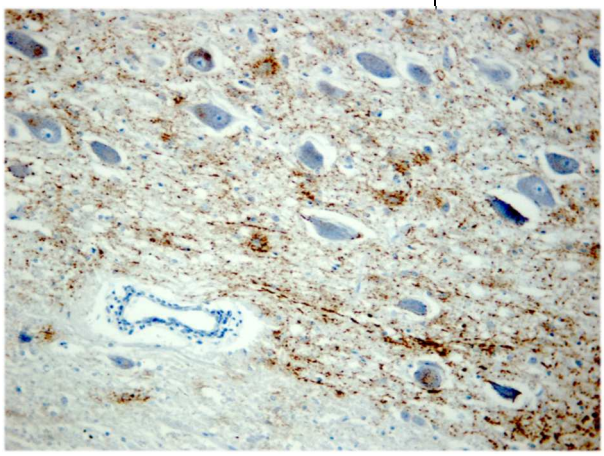


Figure 1. Immunohistochemistry: section of the obex showing the prion protein in the dorsal motor nucleus of the vagus nerve -

2. SPECIFIC SAMPLING REQUIREMENTS

(to fulfil the current statutory requirements as laid down in Annex X to regulation (EC) No 999/20001)

These guidelines are based on the approaches recommended in the OIE manual chapters for BSE and scrapie
http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.06_BSE.pdf
http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.13_SCRAPIE.pdf

The minimum sampling requirement for any animal from either source population is the **brainstem** (at the level of the obex). In addition, for small ruminants it is advised that **part of the cerebellum** is also collected. Both of these brain areas can be accessed through the *foramen magnum* using a proprietary sampling spoon (see below for sampling methods).

Cerebellum may also be a useful sample to assist with confirmation of the classification of atypical bovine TSE[1].

The quantity of tissue taken for testing should be sufficient to provide the following:

[1] Konold T, Bone GE, Clifford D, Chaplin MJ, Cawthraw S, Stack MJ, Simmons MM (2012) Experimental H-type and L-type bovine spongiform encephalopathy in cattle: observation of two clinical syndromes and diagnostic challenges. BMC Vet Res 8:22

- **A hemisection[2] of fresh brainstem** at the level of the obex, **for the initial rapid test** *(or a full transverse section immediately adjacent to the obex, or equivalent sample taken unilaterally using one of the commercially available sampling devices).*

- **A fixed hemisection of brainstem** at the level of the obex **for confirmatory Immunohistochemistry (IHC) and Histopathology (HP)**. *(Ideally this sample would be fixed immediately, but for practical reasons it is appropriate to keep the sample chilled, and fix it on receipt of a positive rapid test. In some countries it is established practice to freeze material prior to fixation. While this does not detract from the subsequent immunoreactivity of positive cases, it can significantly compromise the tissue morphology, making the contextual interpretation of negative or equivocal immunostains difficult or impossible).*

- **A fixed section of cerebellum** may be required for the confirmation of atypical scrapie using IHC.

- Sufficient remaining **fresh-frozen medullary tissue** (adjacent to the obex) **and fresh-frozen cerebellum** to provide fresh material **for confirmatory Western blot and primary molecular testing (discriminatory WB) and possibly a range of secondary and tertiary testing** (5-10 g whenever possible).

- Brain tissue from other areas in addition to the brainstem and cerebellum are always an advantage, as are lymphoreticular tissues when available. Although not essential for statutory purposes, they will be important in

[2] Historically, a full transverse cross-section of the brainstem at the level of the obex has been recommended for histopathological confirmation. With the advent of rapid molecular techniques, the need to apply multiple tests in a single animal has led to competition between tests for the optimal early diagnostic sites at the obex. Hemisection of the brainstem at the level of the obex will result in loss of the ability to assess the symmetry of lesions, but the need for such assessment is less if immunohistochemistry is used. If this approach is adopted however, it becomes critical to ensure that the target site is not compromised. The dorsal nucleus of the vagus nerve (the optimal target area for most cases of scrapie) is small, and lies close to midline

characterizing the scrapie strain present if the results differ from the expected confirmation at the level of the obex

3. BRAINSTEM AND CEREBELLUM SAMPLING METHOD

Whole brain removal is ideal. However, this is not always practical or achievable and the following approaches outline how to remove the **brainstem and cerebellum** through the *foramen magnum*.

Any method that produces the entire brainstem intact and uncontaminated may be used. The samples should be collected in a manner that prevents any risk of contamination between successive samples.

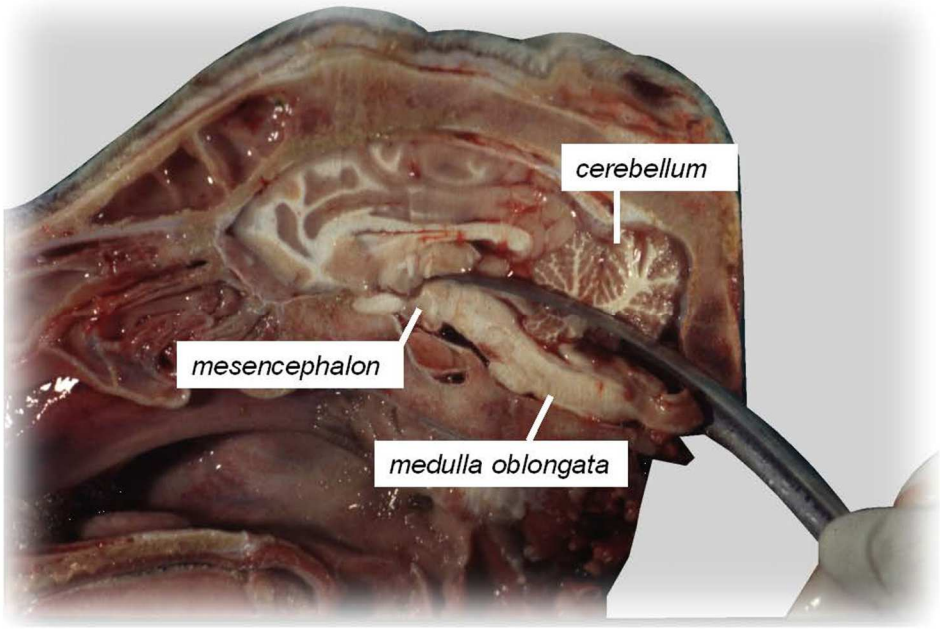


Figure 2. Sheep head split mid-sagitally to demonstrate how the sample should be collected through the *foramen magnum* using the metal spoon

3.1. EURL Method

- Place the disarticulated head upside down on table with the foramen magnum facing the operator (Figure 3). Remove any blood clots obscuring the view of the

brainstem, and identify the dura mater. Use a clean pair of scissors to reflect the dura mater and expose the brain stem.

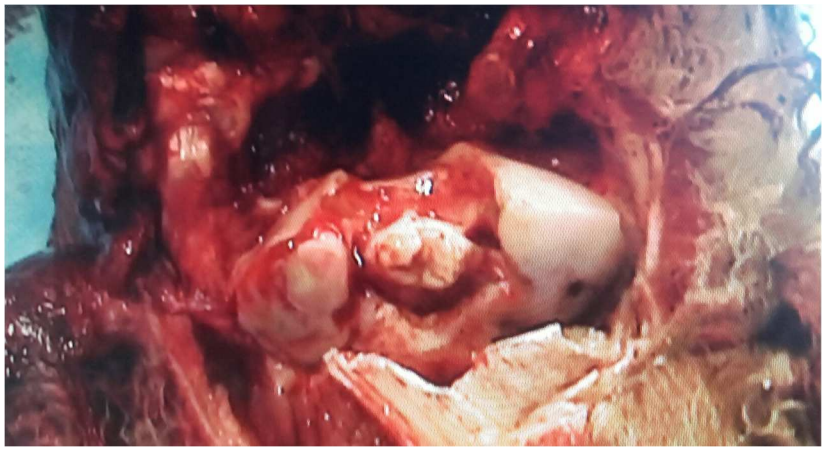


Figure 3: Foramen magnum is visible after the disarticulation of the head

- Gently hold the brain stem as close to the end as possible with a new/clean pair of forceps and move the brainstem to the side to visualize the cranial nerves. Insert the scissors into the foramen magnum and cut through the cranial nerves (VII –XI). Do this on both sides of the brainstem, taking care not to damage it (Figure 4).

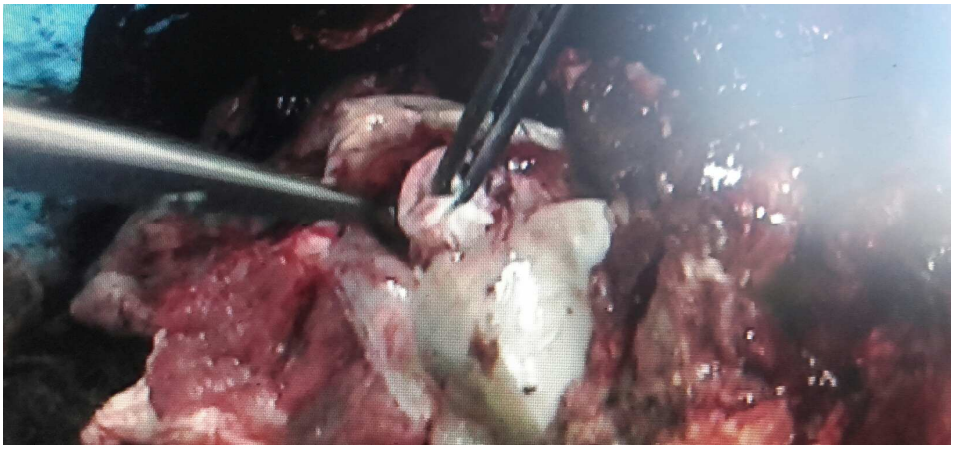


Figure 4: Visualisation of cranial nerves

- Using the forceps, very gently pull the brainstem until it is straight and insert the spoon above the brainstem with the cutting edge of the blade

facing down. Keep the blade of the spoon against the bone above as it is inserted. Insert to the level of the notch of the spoon –approximately 8-10 cm. When in position, point the cutting edge of the blade downwards by lifting the handle upwards to sever the brainstem.

- Cut through the cerebellar peduncles by moving the handle from side to side.
- After completing the cutting, carefully withdraw the spoon and insert it with the blade pointing downwards on top of the brainstem until it enters to a depth of 8-10 cm. Gently move the blade side to side and downwards, cutting through the rostral medulla (avoiding rotational movement as this will damage the obex).
- Gently pull with the forceps and using the spoon as a scoop pull as much brainstem as possible out of the skull. If resistance is encountered, continue to cut through the brainstem with a side to side and downward motion of the handle (Figures 5 and 6)

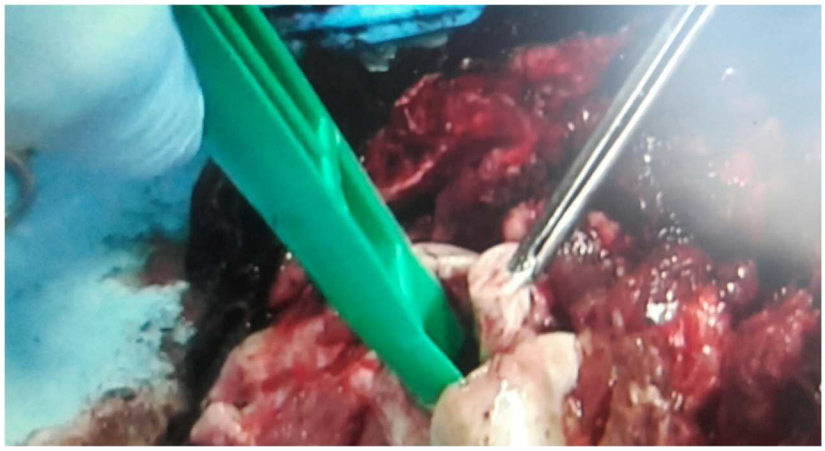


Figure 5: Use of forceps and spoon to extract the brainstem

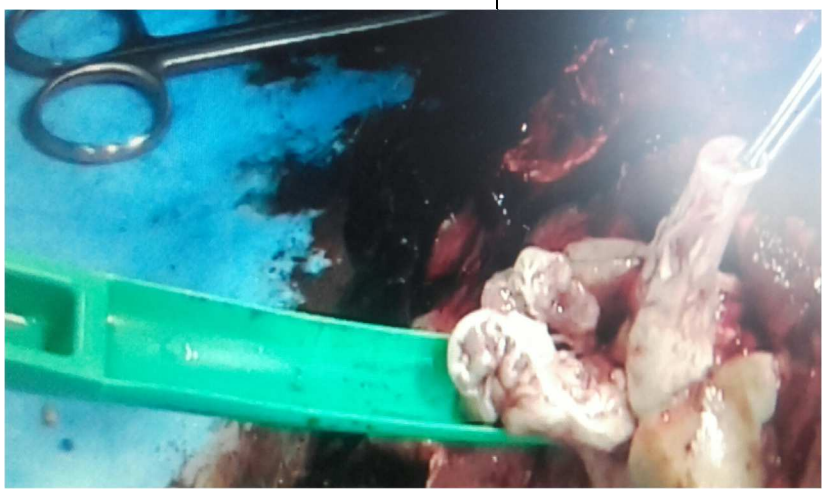


Figure 6. Brainstem extraction from *foramen magnum*

- If necessary, with the head still upside down on the table, look downwards through the foramen magnum and identify the cerebellum. Insert the spoon underneath the cerebellum and lever it upwards into the centre of the space vacated by the brainstem. If the cerebellum is not moving freely, loosen it by moving the spoon gently around underneath it. Lift the cerebellum towards the foramen magnum using the spoon to support and guide, and gently take hold of the cerebellum through the foramen with the forceps. Remove the cerebellum through the foramen magnum.

4. ISSUES RELATED TO SUBSAMPLING FOR SCREENING TESTS

Rapid tests protocols for active surveillance of healthy slaughter and/or at risk populations generally require a specific weight of brainstem tissue, taken at the level of the obex, to be presented to the test.

In cattle BSE the pattern appears to be highly consistent, with early changes appearing first in certain nuclei in the brainstem at the level of the obex. Following experimental oral challenge with BSE, the earliest visible PrP accumulation is consistently seen (using immunohistochemistry - IHC) in the nucleus of the solitary tract, with involvement of the adjacent dorsal motor nucleus of the vagus nerve (DMNV) and the nucleus of the spinal

tract of the trigeminal nerve following soon after. The vestibular nuclei in the rostral medulla may also become involved at an early stage. The IHC patterns observed in many 'early' field cases (detected through both active and passive surveillance) support the consistent early involvement of these areas in natural disease. These areas are recognised as the diagnostic target areas for sampling (Figure 7)

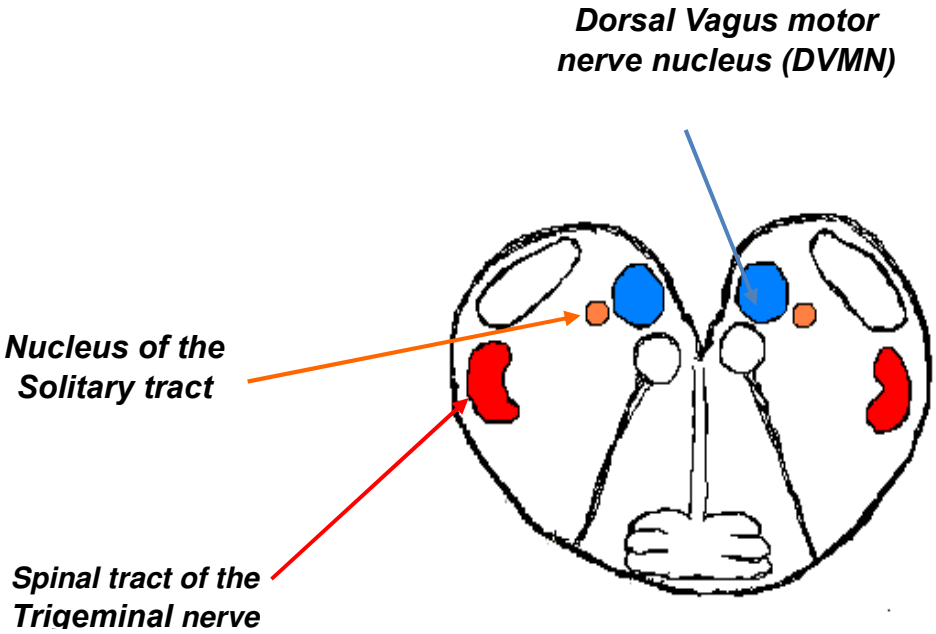


Figure 7: Target areas for sampling

4.1. Sampling 'tools'

The EURL has a role in evaluating and approving rapid test kits for use within the EU. Sampling tools are sometimes offered as part of a commercial test package, and as such

have to be evaluated as fit for purpose, in particular to ensure that any tissue disruption as a result of sampling does not compromise the suitability of material for confirmatory testing should it be required.

In principle these tools take a core of grey matter from the obex region of the brainstem thereby reducing any dilution effect of the peripheral white matter and increasing the sensitivity of the test. If used correctly, and with a clear understanding of the three-dimensional anatomy of the TSE target areas, these tools perform adequately and dispense with the need to assess the weight of each individual sample, with considerable savings in time. However, there are a number of potential drawbacks with this technique that users should be aware of and conduct appropriate monitoring checks.

These drawbacks can be broadly divided into two categories:

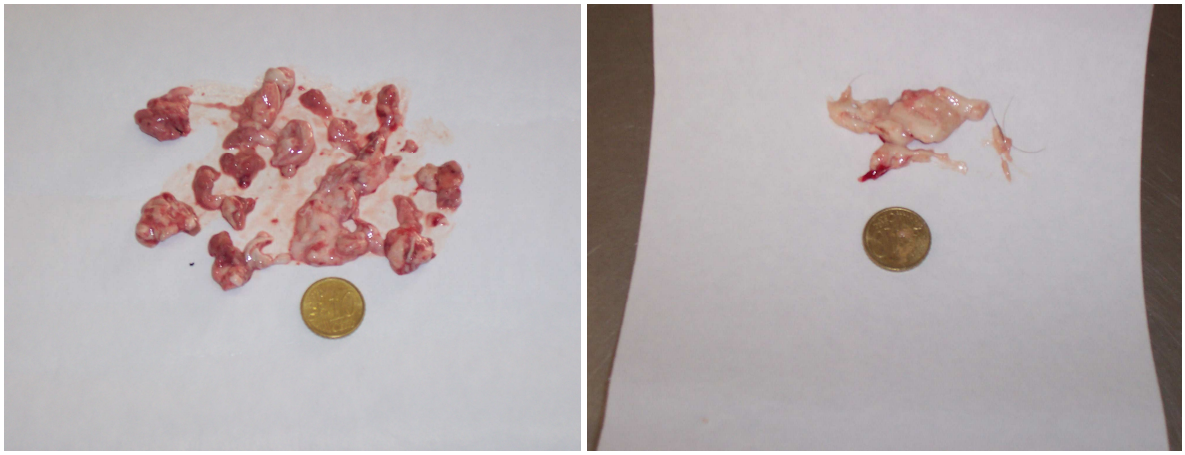
- Inadequately sampled brainstem delivered to the laboratory.

This can be a problem, especially when dealing with fallen stock, where material may be significantly autolysed before sampling takes place. In such cases, the obex region may be damaged or incomplete in some way (Figure 8) or the sample may be so distorted or autolysed that anatomical orientation is not possible (Figure 9).

In these cases, it will not be possible to take an anatomically targeted sample. This cannot be avoided, but it should be recorded, to assist with interpretation of the resulting test results, and to feed back to the personnel collecting the samples that such poor-quality samples should be avoided whenever possible.

Figure 8: Damages of the obex region

a) Sampling damage: incomplete tissue integrity compromised



b) Autolysis – obex identifiable

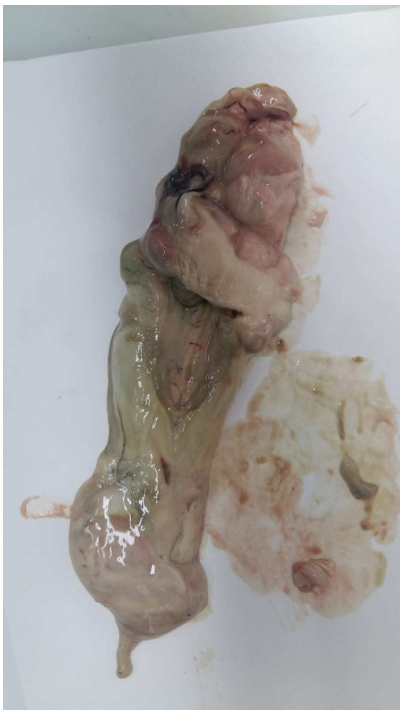
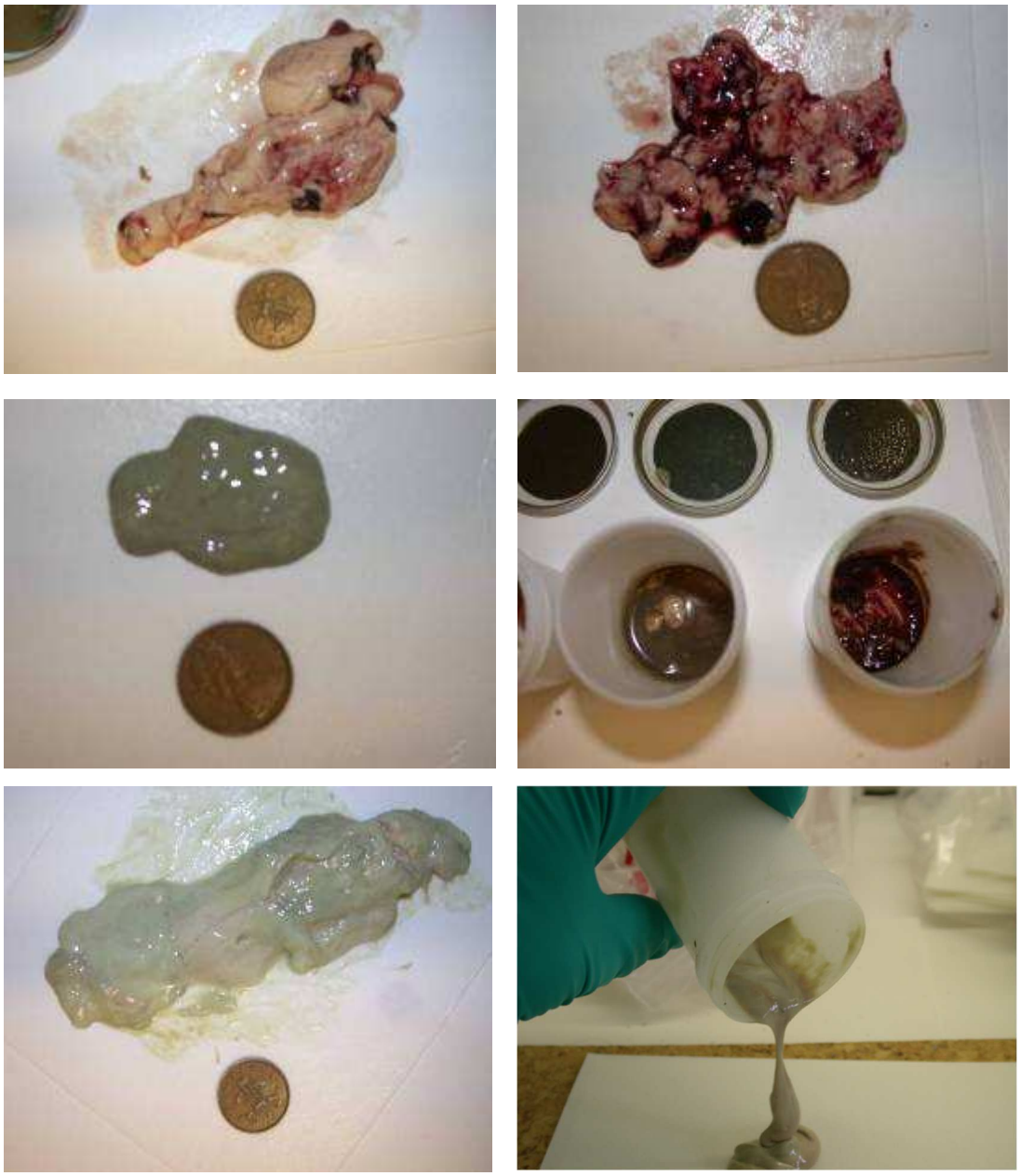


Figure 9: Distortion of brainstem – obex not identifiable



This problem affects both tissue slice and sampling tool samples.

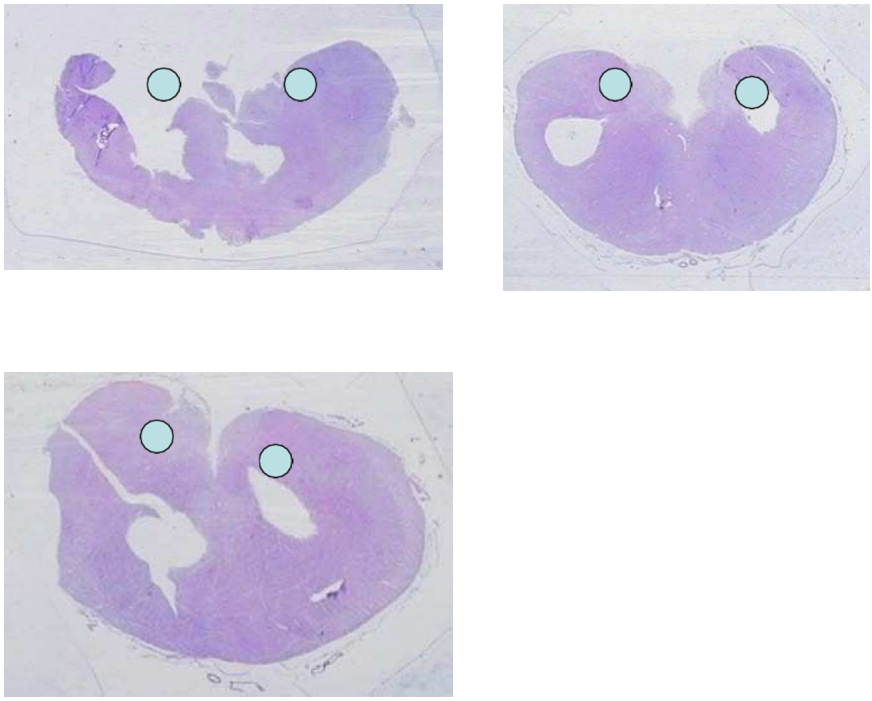
- Inappropriately targeted sample in early/ pre-clinical disease

To avoid compromising the sensitivity of the test, the sampler must ensure that the relevant TSE target areas are adequately represented, in addition to the sample being of a consistent and appropriate weight for the test.

The nucleus of the solitary tract, which is the earliest site of PrP accumulation at the obex in pre-clinical disease in cattle, runs rostro-caudally throughout the caudal brainstem but in its most caudal portion (caudal to the obex) it runs more medially, immediately adjacent to the spinal canal, and dorsal to the parasympathetic nucleus of the vagus nerve.

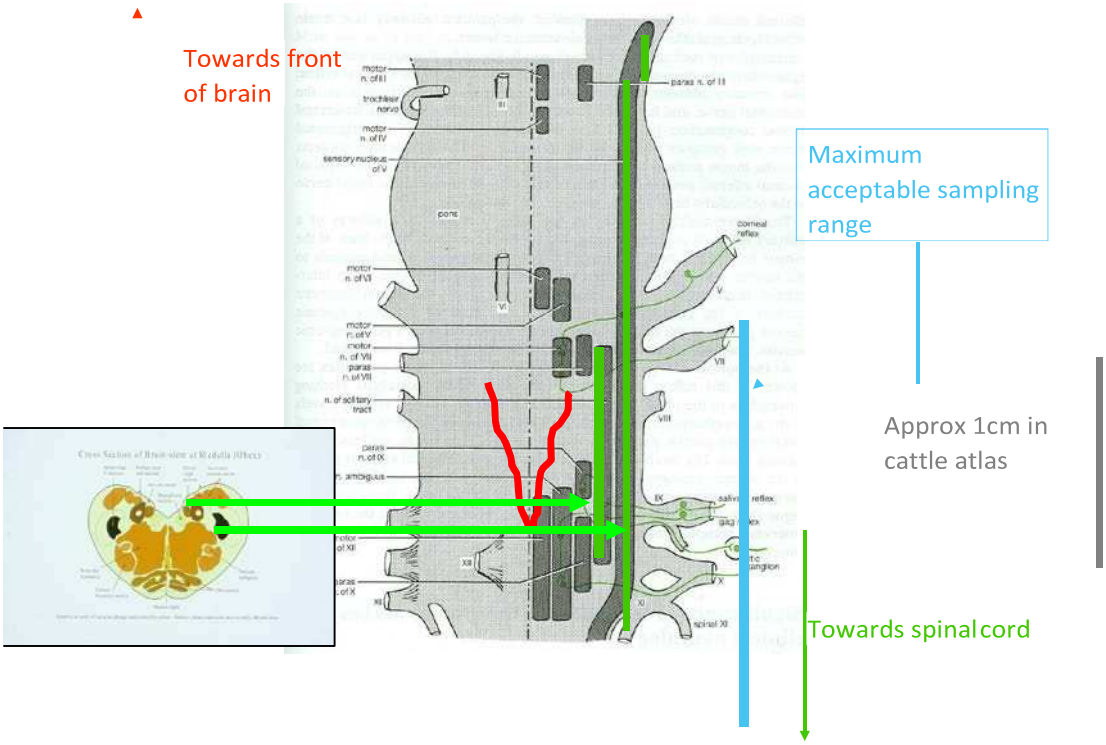
One potential danger with syringe-type 'core' extractor samples taken from variable distances caudal to the obex is that the resulting sample may be too caudal to incorporate the nucleus of the solitary tract at the entry point, and may potentially miss the target areas more rostrally if the sampling tract veers laterally or ventrally (Figure 10).

Figure 10: Inappropriate sample site using a sampling syringe. (Blue circles represent the position of the nucleus of the solitary tract)



This presents a potential sensitivity problem, especially in active surveillance cases where the PrP accumulation is likely to be very focal, and anatomically targeted to the nucleus of the solitary tract, the dorsal motor nucleus of the vagus nerve and the nucleus of the spinal tract of the trigeminal nerve (Figure 7). Such cases may be missed by any sampling method that takes a longitudinal core which, if inaccurately directed, might not contain the target areas.

Figure 11: Target areas



Robust training and great care are needed in the application of this type of sampling method to ensure that the *initial rapid test* is not compromised by the collection of a sample which does not represent the desired target area.

All of the above issues apply also to small ruminants, with the added complication that PrP distribution patterns vary more than in cattle, and the physical size of the brainstem is smaller than in cattle.

The principal target area (the dorsal nucleus of the vagus) lies very close to midline, and any approach which relies upon hemisectioning through the midline has little tolerance for inaccuracy.

Accepting that this should be a **secondary option only,** where the obex is not identifiable, other parts of the brainstem/cervical spinal cord can be tested using approved rapid tests, even though their approval is currently specific to testing of the obex. In this case a positive result is valid and should be reported as such. Negative results must however be reported with the caveat that optimal tissues were not available for testing. The alternative option is to report such tissues as "no test" i.e. un-testable as the target tissue is not available.

5. ACTION ADVISED

Each NRL should ensure that all local sampling instructions contain appropriate detailed reference to the cross-sectional and longitudinal anatomy of the structures which require to be targeted.

Additionally, instructions must be included on how to sample material which is not optimally collected/oriented or properly identifiable at an anatomical level when it is not possible to correctly position the sampling tool, or accurately identify the obex.

It is advised that the accuracy of sampling is monitored by the NRL by review of a randomly-selected proportion of negative cases in addition to any positives which are referred for confirmation.

This document is largely based on a previous one that originally was made available by APHA (UK) as EURL for TSEs. After the transition of the EURL to our consortium, in the documentation that we are making available, some minor changes were needed to update information regarding e.g. logistical aspects, sample flow, contacts and references, or changes carried out by the relevant companies in protocols and nomenclatures.