

## ANNEX 1

# CONSUMPTION OF BOVINE TONGUE AND THYMUS: A RISK TO PUBLIC HEALTH?

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## ABSTRACT

**In order to minimise the risk to public health that could arise from the consumption of BSE-infected food, several measures such as the removal and destruction of specified risk material (SRM), which includes the tonsils, have to be implemented.**

**The present paper describes the exact anatomical location of the bovine lingual tonsil and demonstrates that the currently prescribed technique for harvesting bovine tongues in the slaughterhouse is not appropriate for removing all SRM. We therefore propose a curved incision starting three cm rostral to the most caudal vallate papilla and undermining the lingual mucosa up to the level of this papilla. To sever the tongue from the head, this incision is followed by a transverse cut as far as the lingual process of the hyoid bone.**

**Additionally, we present some data about calf thymuses from which we can deduce that their consumption is safe for public health.**

## INTRODUCTION

Since mad cow disease raised its head in the United Kingdom in 1985 (Anderson *et al.*, 1996), interest in transmissible spongiform encephalopathies (TSE) has increased enormously, not only among scientists but also in the various sections of the media (Beisel and Morens, 2004). One important reason for this is the fact that the new variant Creutzfeldt-Jakob disease (vCJD) in humans has been linked to the occurrence of Bovine Spongiform

Encephalopathy (BSE) in cattle and, more specifically, to the consumption of BSE-contaminated food (Bruce *et al.*, 1997; Hill *et al.*, 1997; Zeidler and Ironside, 2000; Narang, 2001; Aguzzi *et al.*, 2003). Despite the fact that the BSE epidemic in the United Kingdom had passed its peak after 1992 (Beghi *et al.*, 2004), there was a very great fear of BSE on the European continent, particularly around the turn of the century, and when at the beginning of 2005 a case of BSE in a goat was confirmed (European Commission, 2005) emotions flared up again.

The first cases of vCJD in humans were diagnosed ten years after the outbreak of BSE (Beghi *et al.*, 2004). It was therefore estimated that the incubation period of vCJD in humans is at least five years, but the mean and maximum incubation periods are still not known (Knight, 2003). In the same way as scrapie in sheep (Dawson *et al.*, 1998; Hunter, 1998; Hunter and Cairns, 1998), the susceptibility of humans to vCJD is determined by the genotype (Wadsworth *et al.*, 2004; Mitrova *et al.*, 2005). This is because there is polymorphism at codon 129 of the gene coding for the prion protein. This codon can code for the amino acids valine or methionine. In a healthy population the majority of people are heterozygotic, whereas the victims of vCJD are predominantly homozygotic for methionine (Ironside and Head, 2004). Wadsworth *et al.* (2004) therefore believe that the human prion protein with valine 129 prevents the expression of vCJD. It therefore appears that, just as in sheep, there are resistant and susceptible genotypes in humans as well. The following quotation by Brown (2005) should certainly be mentioned in this context: “Considering the disease’s incubation period, which tends to vary with human genotype, the possibility of a second wave of vCJD cases cannot be excluded, but I do not believe this will occur. There will, however, be other cases.” Brown (2005) suggests that genetically susceptible humans exposed to prions have already become victims of vCJD, while in resistant subjects the disease will only develop after many years, or not at all, because of the much longer incubation period.

TSEs are characterised by an accumulation and replication of the disease-specific isoform PrP<sup>Sc</sup> of the endogenous prion PrP<sup>C</sup> (Prusiner, 1998) in nerve tissue and lymphoid tissue, such as tonsils and thymuses (Fournier, 2001; Aguzzi *et al.*, 2003). The development of TSE in humans occurs primarily as a result of the consumption of prion-infected tissues from cattle and small ruminants. However, in view of the fact that oral infection is relatively inefficient, the risk of infection is greater when the tissues consumed contain more prions (Huang and MacPherson, 2004). In the consumer the ingested prions accumulate predominantly in tonsils and Peyer’s patches which are particularly suitable for antigen uptake, partly because of the presence of M cells (Aguzzi *et al.*, 2003). After neuro-invasion the prions reach the central

nervous system where neuronal vacuolation and a loss of neurons occur (Knight, 2003; Press *et al.*, 2004). This process results in the typical spongy appearance of the brain. Other histological changes are astrocytosis and the formation of amyloid plaques (Beisel and Morens, 2004). The outcome is a progressive, fatal neurological disease characterised predominantly by mental regression (Narang, 2001).

In view of these implications of BSE for public health, TSE surveillance was instituted in 1990 in the United Kingdom and became operational in the whole European Union from 1993. A rapid BSE test was developed and this had to be administered to all slaughtered cattle over 30 months of age (Venturini *et al.*, 2000; European Commission, 2003). Emergency slaughter represent an exception to the rule; in such cases animals have to be tested from the age of 24 months. The term specified risk material (SRM) was also introduced. This is high-risk material which is suspected of constituting a serious risk to human or animal health even after heat treatment (European Commission, 2003). As far as the head is concerned, the skull, with the exception of the mandible but including the brain and the eyes, of cattle over six months of age (Portugal and Great Britain) or over 12 months of age (other EU member states) is classified as SRM. Irrespective of the age of the cattle, the tonsils are always classified as SRM (European Commission, 2003). Bovine tonsils form a ring of lymphoid tissue around the entrance to the pharynx, also known as Waldeyer's ring (von Waldeyer-Hartz, 1884; Perry and White, 1998). Domestic animals have six tonsils: the lingual tonsil, the palatine tonsil, the para-epiglottic tonsil, the pharyngeal tonsil, the tubal tonsil and the tonsil of the soft palate (Cocquyt *et al.*, 2005). The relative size of the tonsils varies from species to species. In contrast to the tonsils, the bovine thymus is only classified as SRM if it comes from animals from Great Britain or Portugal that are more than 6 months old (European Commission, 2003). This regulation suggests that the consumption of bovine thymuses also is not entirely safe. In France the sale of thymuses is in fact forbidden (Anonymous, 2001).

As long as it is free from tonsil tissue, the bovine tongue is not classified as SRM and so is fit for human consumption. In order to fulfil this requirement, Europe (European Commission, 2003) lays down as a guideline that bovine tongues must be removed in the slaughterhouse by a transverse incision rostral to the lingual process of the basihyoid bone. According to Kühne *et al.* (2005) and Wells *et al.* (2005), however, this method does not prevent lymphoid tissue from occurring in tongues fit for human consumption. To ensure the complete removal of the lingual tonsil, its anatomical location must be described exactly and the incision used in the slaughterhouse adapted accordingly. According to Barone (1997) the lingual tonsil is situated at the root of the tongue, more precisely in the section of tongue

caudal to the vallate papillae, rostral to the epiglottis and between the palatoglossal arches. It consists of an aggregation of both primary and secondary lymphoid follicles arranged around epithelial crypts (Ackerknecht, 1943; Habel, 1975, 1992; Manesse *et al.*, 1995).

This study describes in detail the location of the bovine lingual tonsil with a view to critically examining the current European guideline for the excision of bovine tongues and presenting an excision method that guarantees maximum removal of tonsillar tissue. As calf thymuses can also contain prions, they might constitute a risk to public health. This is clearly expressed in the international legislation. This article discusses the origin and morphology of thymuses and on the basis of a literature search relating to the spread of prions investigates whether the consumption of thymuses is safe or not.

## MATERIAL AND METHODS

A total of 15 tongues from adult cattle were collected immediately after slaughter. Samples of epithelium and underlying tissue were taken from ten bovine tongues. The area delineated by the caudal vallate papilla and the epiglottis was sectioned over the whole width of the tongue into pieces 2 cm wide, 1 cm long and 1 cm high. These sections were fixed for seven days in 3.5% formaldehyde, after which they were embedded in paraffin and formed into blocks. One 8  $\mu$ m thick section from each block was stained with haematoxylin (Haematoxylin (C.I. 75290), Merck KGaA, Darmstadt, Germany) and eosin (Eosin yellow (C.I. 45380), VWR international bvba/sprl, Louvain, Belgium).

A rectangular dorsal piece was dissected out from four bovine tongues over the full width from the caudal vallate papilla to 5 cm rostrally. This sample was cut into smaller pieces, which were clarified in similar fashion to that described above for histological examination. Sections 8  $\mu$ m thick were made from each block at intervals of 1 mm and stained with haematoxylin and eosin.

A sample of the root of the tongue was taken from the remaining bovine tongue for identification of the openings visible on the tongue surface. Negrosine was injected into the openings with the aid of a blunt 25-gauge needle. A histological section was prepared as described above. Another sample, also of the root of the tongue, was fixed for several days in a HEPES-buffered solution of 2% paraformaldehyde and 2.5% glutaraldehyde. The sample was then fixed for two hours in 1% unbuffered osmium tetroxide followed by dehydration with increasing percentages of alcohol and acetone. This sample then underwent critical point

drying. Finally it was mounted on an aluminium plate, coated with platinum and examined under a scanning electron microscope (JEOL JSM 5600 LV, JEOL, Brussels, Belgium).

The light microscopic studies of all HE-stained tissue sections were performed using a motorised microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium) connected to a digital camera (Olympus DP 50, Olympus Belgium, Aartselaar, Belgium).

## RESULTS

The root of the tongue has a rough surface and numerous small, but macroscopically visible openings are arranged more or less in rostralateral grooves. The most rostral openings are found approximately 2 cm caudal to the most caudal vallate papilla (Figs. 1 and 2). Histological examination after injection of nigrosine in the openings and scanning electron microscopic examination of this area identified the openings as tonsillar fossulae, which are the openings of the crypts of the tonsillar follicles (Figs. 3, 4, 5 and 6).

The tissue under the keratinised squamous epithelium of the root of the tongue consists primarily of loose connective tissue, adipose tissue, striate muscle tissue and salivary glands. The subepithelial connective tissue contains large quantities of lymphoid tissue arranged in primary and secondary lymph follicles, but diffuse accumulations of lymphocytes are also frequently observed. Lymphoid tissue is found over the whole width of the root of the tongue, but is particularly profuse on the lateral side of the dorsal tongue surface.

Most lymph follicles in the root of the tongue are arranged into tonsillar follicles (Fig. 4), which are surrounded by a thin capsule of compact connective tissue that forms the border with the surrounding fatty tissue and the salivary glands. The tonsillar follicles are up to 5 mm in height and have a diameter of 3 mm. The crypts are 2 to 2.5 mm deep. The crypt openings with a diameter of 0.5 mm are 10 times larger than the openings of the salivary glands. The crypt epithelium is in some places transformed into reticular epithelium that is massively infiltrated by lymphocytes (Figs. 3 and 4).

Various lymph follicles organised into aggregations without crypts are observed between the tonsillar follicles of the root of the tongue. Usually they are surrounded by compact collagenous connective tissue without having contact with the neighbouring superficial epithelium. However, this was not the case in two bovine tongues studied, where the superficial epithelium was massively infiltrated by lymphocytes.

Only a few primary follicles and diffuse accumulations of lymphocytes can be seen in the subepithelial tissue 3 cm rostral to 2 cm caudal to the most caudal vallate papilla. These structures are not encapsulated and are frequently in contact with the overlying superficial epithelium, where this epithelium is usually infiltrated by lymphocytes (Fig. 7). This part of the lingual lymphoid tissue can only be detected by histological examination. No further lymphocyte aggregations can be found rostral to this area. Figure 8 gives a schematic overview of the distribution of tonsillar tissue in the root of the bovine tongue.

## DISCUSSION

Given that bovine tongue as such is not classified as SRM but contains a tonsil (the lingual tonsil) that by definition is classified as SRM, the question recently arose as to how far bovine tongues are safe for human consumption. Although the Spongiform Encephalopathy Advisory Committee (SEAC) concluded that the maximum exposure of humans to infectious tongue tissue is very low (Anonymous, 2004), we wish to adopt a more nuanced approach. This is because our study has shown that the lingual tonsil is not confined to the area where crypt openings are found. We have in fact been able to demonstrate lymphocyte aggregations that were not associated with a crypt. Kühne *et al.* (2005) have already proposed excising bovine tongues in the slaughterhouse by making an incision at the level of the most caudal vallate papilla. However, this is insufficient because subepithelial lymphocyte aggregations still occur in the area located rostral to these papillae. A curved incision starting 3 cm rostral to the most caudal vallate papilla and undermining the lingual mucosa to the level of the most caudal vallate papilla, followed by a transverse incision as far as the lingual process of the hyoid bone can completely remove the lingual tonsil (Fig. 9). This method should combine the maximum removal of the lingual tonsil, and thus a maximum reduction in the risk to public health, with minimal loss of lingual muscle tissue, which is economically advantageous.

The need to excise the bovine tongue correctly and possibly even to completely ban it from the food chain is strengthened by the finding that in intracerebrally infected hamsters prions can be transported from the central nervous system to the tongue via the sensory lingual nerve or the motor hypoglossal nerve (Bartz *et al.*, 2003). Infectious prions have been shown to be associated with axons of lingual nerves. Bosque *et al.* (2002), however, found that proliferation of PrP<sup>Sc</sup> can occur in skeletal muscles of mice and that large numbers of

prions can accumulate here. More emphatically still, it has recently even been shown that scrapie prions can occur in the taste papillae of sheep tongues (Casalone *et al.*, 2005). If we were to extrapolate these findings to BSE in cattle, there might possibly be considerable exposure to prions from the consumption of beef, even if this meat is largely freed from nerve and lymphoid tissue. This assumption runs counter to the current belief of the SEAC and obviously requires further investigation and a possible revision of the definitions of “risk material”.

The question may then arise as to whether calf thymuses are safe to eat in view of the fact that the thymus gland is a lymphoid organ and can therefore contain prions (Bellworthy *et al.*, 2005). In cattle, the thymus is highly segmented. It consists of head, neck and chest sections, known as the cervical lobe, intermediate lobe and thoracic lobe (Nomina Anatomica Veterinaria, 2005). The head section consists of a left and a right part. The neck and chest sections, however, are both solitary. A single small strip of tissue connects the neck section to the chest section. The single neck section emerges cranially into two branches that are both connected to the paired head section. Accessory thymuses can also occur (Schummer *et al.*, 1984).

A thymus from a newborn calf weighs about 80 to 100 grams. After 6 weeks the thymus weight increases to 400 g (Berg, 1995) and its maximum weight, varying from 300 to 900 g, is reached at about the age of 9 weeks, when the calves weigh approximately 100 kg (T. Thibaut, personal communication). Thymus involution then starts from this age (Martin and Schauder, 1938). This is due to the sudden increase in sex steroid concentrations and the increased production of growth hormone at the beginning of puberty (Montecino-Rodriguez *et al.*, 2005). Involution begins in the head section and the small strips of tissue between the head, neck and chest sections (Schummer *et al.*, 1984). At the age of 15 weeks the calves weigh between 150 and 200 kg (T. Thibaut, personal communication) and the thymus weight has by then already fallen to 300 g (Barone, 1996). The two branches now separate off from the neck section so that the thymus consists of four separate parts: the two separate branches, the single neck section itself and the single chest section (Schummer *et al.*, 1984). After the neck section has also disappeared, the chest section can still be detected for six years or longer in the form of thymus fat pads (Schummer *et al.*, 1984).

In Flanders it is above all double-muscle calves and Friesian or red-and-white breed calves that are fattened. The double-muscle calves are usually slaughtered at 32 to 34 weeks when the animals weigh approximately 300 kg. Dairy breed calves are slaughtered earlier, at 26 to 28 weeks. These animals then weigh about 260 kg. The thymuses of these veal calves,

which weigh 200 to 300 g, are sold (T. Thibaut, personal communication). In cattle that develop BSE, the spread of prions to the peripheral lymphoid tissue normally does not occur before the second year of life (Bellworthy *et al.*, 2005) and so the risk to health from calf thymuses can therefore be considered very low. Consequently, under European legislation the thymus is not classified as SRM, except in cattle from Great Britain and Portugal over 6 months old (European Commission, 2003). It may be considered that the consumption of calf thymuses involves no risk to public health in view of the fact that a thymus that is fit for consumption cannot yet contain any prions because of the slow spread of prions to peripheral lymphoid tissue.

## CONCLUSION

The consumption of bovine tongue involves a limited risk to public health because the lingual tonsil is not completely removed by the excision method currently employed. By establishing the exact location of the lingual lymphoid tissue an alternative incision is proposed here that combines the maximum removal of the lingual tonsil, and thus a maximum reduction in the risk to public health, with minimal loss of lingual muscle tissue. Recent studies of the occurrence and spread of prions in laboratory animals and sheep, however, suggest that, even if the bovine tongue is completely free from lymphoid tissue, there is possibly still a certain risk. Action must be taken at European level if we are to continue to guarantee that food of animal origin is 100% safe for the consumer.

Thymuses from veal calves may be eaten with an easy mind because of the late spread of prions to this organ.

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## ANNEX: Figures

Fig. 1. Schematic representation of a bovine tongue indicating the main morphological reference points with, beside it, a photographic image of a preparation available in a supermarket (a: lingual torus, b: area of the vallate papillae, c: area of the tonsillar fossulae).

Fig. 2. Detail of the tongue root in which the vallate papillae (a) and the tonsillar fossulae (b) are clearly visible. The distance (2 cm) between the most caudal vallate papillae and the first row of tonsillar fossulae is indicated by the double arrow. The box shows the sampling site for the scanning electron microscopic photographs (see Figs. 5 and 6) (R = rostral, C = caudal).

Fig. 3. Follicles of the lingual tonsil in which negrosine is clearly present in the crypt. (1) Multilayered keratinised pavement epithelium of the tongue surface, (2) tonsillar follicle crypt, (3) multilayered unkeratinised crypt epithelium, (4) reticular epithelium (infiltrated with lymphocytes), (5a) germinal centre of a secondary lymph follicle, (5b) mantle zone of a secondary lymph follicle, (6) paranodular tissue, (7) compact collagenous connective tissue capsule, (8) salivary gland, (9) salivary gland outlet.

Fig. 4. Tonsillar follicle in which the opening of the crypt is visible. (1) Multilayered keratinised pavement epithelium of the tongue surface, (2) tonsillar follicle crypt, (3) multilayered unkeratinised crypt epithelium, (4) reticular epithelium, (5a) germinal centre of a secondary lymph follicle, (5b) mantle zone of a secondary lymph follicle, (6) paranodular tissue, (7) compact collagenous connective tissue capsule, (8) salivary gland.

Fig. 5. Scanning electron microscopic image of five tonsillar fossulae (arrows) obtained from the boxed area of the root of the tongue illustrated in Fig. 2 (R = rostral, C = caudal).

Fig. 6. Highly magnified scanning electron microscopic image of the central tonsillar fossula from Fig. 5.

Fig. 7. Histological image showing the presence of lymphoid tissue in the area extending 3 cm rostrally to 2 cm caudally to the most caudal vallate papilla. The lingual epithelium (1) is infiltrated in some places by lymphocytes (2) and diffuse lymphocyte aggregations (3) are present in the subepithelial connective tissue.

Fig. 8. Schematic overview of the position and density of lingual tonsillar tissue. The lymphocyte density ranges from high (black) through moderate (dark grey) to low (light grey). Note that tonsillar tissue can occur up to three cm rostrally to the most caudal vallate papilla.

Fig. 9. Median section through a bovine head illustrating the currently used excision technique (dotted line) and our proposed incision (uninterrupted line). The white dot indicates the location of the most caudal vallate papilla, while the arrow points to the lingual process of the hyoid bone.