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OPINION

THE SAFETY OF ANIMAL RENNET IN REGARD TO RISKS FROM ANIMAL TSE AND BSE IN PARTICULAR

ADOPTED BY THE SCIENTIFIC STEERING COMMITTEE

AT ITS MEETING OF 16 MAY 2002

OPINION

BACKGROUND AND MANDATE

Rennet is used in animal and human foodstuffs (including cheese and food supplements) and in medicinal products and for the manufacture of lactose. Rennet can be produced from animals, mostly calves and adult cattle, from plant materials and by genetic engineering. As rennet may be sourced from certain TSE-susceptible animal species (besides using biotechnological processes or certain plant sources), the SSC was invited to prepare a general opinion on the safety of rennet obtained from calves, adult cattle, small ruminants and pigs with regard to animal TSE risks and particularly BSE risks, including those resulting from the method of harvesting, risks from the epithelium, contamination with lymphoid tissues, contamination with feed, feed bans, risk from cross-contaminated feed, and geographical sourcing.

The SSC asked the TSE/BSE *ad hoc* Group to prepare a scientific report on the subject, which could serve as input into the discussions when preparing its opinion. This report is attached. It served as the basis for the opinion hereafter, which considers only rennet produced from animals and particularly calves and adult cattle.

OPINION

In the opinion hereafter the Scientific Steering Committee assumes that animal rennet is derived from materials from animals that are fit for human consumption.

A. RENNET FROM RUMINANTS

The source of any TSE infectivity in rennet is from the abomasum, from TSE-infected feed within its lumen at the time of slaughter or from cross-contamination.

The feed bans that are currently in place in the EU apply equally to bovines and small ruminants and should therefore exclude the presence of TSE-infected feed in the lumen at the time of slaughter. A number of TSE-risk reduction strategies are currently in use in abattoirs in the EU that reduce risks from cross-contamination and should eliminate them if properly enforced.

With regard to the possible abomasum-related risk, the SSC considers:

1. CALF AND OX RENNET[1]

The risk that BSE infectivity is present in the abomasums from calves and adult cattle is negligible in GBR category I countries.

The limited available studies to date indicate that the distribution of BSE infectivity in cattle is very restricted and does not imply the abomasum. Under the conditions of collection and storage specified in the attached report of the TSE/BSE *ad hoc* Group, which imply feed-bans, avoidance of cross-contamination and recovery and processing of the abomasum without intestine

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[1]   The term 'Ox rennet' is used for that rennet produced from adult bovine animals and 'Calf rennet' is used for rennet produced exclusively from calves

part attached to it, and in the light of current knowledge, the BSE risk in rennet derived from the abomasums of calves and adult cattle of any age slaughtered and passed fit for human consumption is negligible also in countries with a geographical BSE risk level above I.

2. RENNET FROM SMALL RUMINANTS

Available data on small ruminants do not provide the same level of confidence regarding the absence of infectivity in the abomasum: on one hand PrP^{Sc} has been detected in the forestomach and abomasum of sheep experimentally infected with BSE, but BSE has on the other hand never been reported to naturally occur in these species.

Scrapie infectivity in the abomasum of scrapie-infected small ruminants is theoretically possible but currently is not believed to pose a risk.

The assessment of the BSE risks from small ruminants has been reported on in several SSC opinions. Should it be probable that BSE is present in small ruminant flocks under field conditions, then the rennet may represent a possible risk. The risk from rennet prepared from these species by a method similar to calf or ox rennet is therefore not negligible if the presence of BSE-infected feed in the alimentary tract of small ruminants (sheep and goats) is possible (e.g., because of feed-bans) or if TSEs in small ruminants have been proven to occur in the country of origin.

3. CALF RENNET USED IN THE PRODUCTION OF LACTOSE FROM WHEY, AND THEN USED IN MEDICINAL PRODUCTS.

The SSC confirms its statement of 5 April 2002 sharing the conclusion of 27 February 2002 of EMEA's Committee for Proprietary Medicinal Products (CPMP) and its Biotechnology Working Party that the BSE risk in pharmaceutical grade lactose is negligible when rennet is sourced from the abomasum of bovine calves that are fit for human consumption and produced according to the steps as referred to in the above EMEA report of 11-13 February 2002.

4. DERIVATIVES FROM RENNET-TREATED MILK

It follows from the above that derivatives from rennet-treated milk as defined in the attached report, including cheese derived from curds and lactose lactulose, galactose and ethanol derived from whey all have a similar negligible BSE risk, provided no other animal-derived product is used during their manufacture.

B. OTHER SOURCES OF RENNET

5. RENNET FROM PIGS

Provided that BSE-infected feed is not present in the alimentary tract of pigs at slaughter any risk in rennet prepared in a similar manner is negligible.

6. RENNET FROM NON-ANIMAL SOURCES

Rennet from non-animal sources present no risk from TSE so long as no other animal derived material is added.

REPORT ON:

THE SAFETY OF ANIMAL RENNET IN REGARD TO RISKS FROM ANIMAL TSE AND BSE IN PARTICULAR

FINALISED BY THE TSE/BSE AD HOC GROUP

AT ITS MEETING OF 2 MAY 2002

SUBMITTED TO THE SCIENTIFIC STEERING COMMITTEE

AT ITS MEETING OF 16 MAY 2002

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I. MANDATE

The SSC was invited to prepare an opinion on the safety of rennet obtained from calves, adult cattle, small ruminants and pigs with regard to animal TSE risks and particularly BSE risks, including from the method of harvesting, risks from the epithelium, contamination with lymphoid tissues, contamination with feed, feed bans, risk from cross-contaminated feed, and geographical sourcing.

The SSC asked the TSE/BSE *ad hoc* Group to prepare a scientific report on the subject, which could serve as input into the discussions when preparing its opinion.

The current report was adopted by the TSE/BSE *ad hoc* Group at its meeting of 2 May 2002 on the basis of a draft prepared by Dr.R.Bradley**.**

II. REPORT

II.1. BACKGROUND INFORMATION ON RENNET

Rennet is an extract from the fourth stomach (abomasum or rennet-bag) of ruminant animals, principally calves and adult cattle, with the capability of clotting milk by enzymic action. The dictionary definition[2] is: 'any means of curdling milk, especially a preparation of calf's stomach'. The French name for rennet is présure.

The stomachs of lambs, kids, pigs and hares have been used to extract rennet but the principle species used is cattle (calves and adults) (National Dairy Council, 1992). The enzymes extracted from abomasums are chymosin and pepsin and these are produced in glandular cells in the mucosa.

Rennin is the colloquial name given to the enzyme extracted from stomach that coagulates milk. Alternative substances used to clot milk have a vegetable origin or are manufactured from non-animal sources. Vegetable sources include the cheese-rennet herb, 'Ladies bedstraw (*Galium verum*). Fungal enzymes produced by *Rhizomucor miehei, Mucor pusillus and Cryphonectria parasitica* are also available for clotting milk. These are less sensitive to temperature changes than rennet derived from cattle abomasums. Clotting agents prepared from other vegetable material such as figs, pineapple or bacteria, are less successful rennet substitutes. Genetically engineered chymosin (the main enzyme present in calf rennet) has been produced from yeast (*Kluveromyces lactis*), bacteria (*Escherichia coli)* and fungi (*Aspergillus niger*).

In 1874, a Danish chemist, Christian Hansen founded a laboratory in Copenhagen and commenced the first commercial production of rennet from calf and adult cattle stomachs. A factory, trading under the name Chr Hansen Laboratories, was opened in Great Britain (GB) in 1918 and played an important role in the cheese making industry. Several other laboratories in other countries also trade under the name of Chr Hansen Laboratories.

In the 1960s a large increase in cheese manufacture and a deficit in animal-derived rennet stimulated the demand and use of vegetable-derived rennet.

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[2] Chamber's English dictionary, 7th edition 1989

Subsequently advances in genetic engineering led to the use of cloned chymosin prepared in this way from *Aspergillus niger*. In Great Britain, for example, currently at least three companies produce rennet from non-animal sources. Genetically engineered (GE) rennet dominates the market for cheese making in GB. This is largely because animal-derived rennet is more expensive to produce and demands a higher price. So far as is known, no animal-derived material is used in the preparation of either GE or plant-derived rennet and cheeses manufactured using these types of rennet are acceptable for consumption by vegetarians.

Rennet is mainly used in hard cheese making and little is used in the manufacture of soft cottage cheese or fromage frais. Lactose (milk sugar), galactose, lactulose and ethanol are by-products of cheese manufacture derived from whey. Whey is the liquid end-product produced when rennet is added to whole milk, partially skimmed or skimmed milk (milk from which the butter fat/cream is removed by centrifugation). Lactose prepared from whey has an important usage during the manufacture of pharmaceutical and biological products and is also used in confectionery. According to lactose manufacturers, approximately 90% of lactose for use in the pharmaceutical industry is prepared from rennet-derived whey (EMEA, 2002). Whey is used in feed for pigs, possibly other animals and at least historically in infant foods. The other major product of rennet-treated milk is the curd from which cheese is manufactured. A small amount of rennet is sold for cooking.

After milk is treated with rennet 50-90% of it partitions with the whey and 10-50% with the curds where it continues to function duration maturation of the cheese.

Terminology, age of source animals and enzyme composition

The source of abomasum for the production of rennet is either the bovine calf or ox (adult bovine slaughter animal). Calf abomasum is known in the trade as vell and adult abomasum as reed. There is no specification for breed, sex, dairy or beef type.

Vells are collected from newborn calves or milk-fed calves used for veal. The precise cut-off age is not specified but is generally regarded as being six months of age. The product from such vells is called 'Calf rennet'.

Reeds (termed **ox reeds)** are collected from adult cattle reared and sold for beef but including cows. The product from ox reeds is called 'Bovine rennet'.

> **NOTE:** *Caution should be exercised in the use and understanding of the term 'Bovine rennet'. The trade uses the term exclusively for bovine rennet produced from the reeds of adult animals. The casual reader should not assume that 'Bovine rennet' could include rennet produced from calves. It does not. Such rennet is called 'Calf rennet' though it has a bovine origin.*
>
> *To avoid confusion in this report the term 'Ox rennet' is used for that rennet produced from adult bovine animals and 'Calf rennet' is used for rennet produced exclusively from calves. Where it is necessary to refer to all rennet produced from bovine animals the term 'Calf and ox rennet is used'.*

In 2002 (by contrast with 1989), more rennet is produced from non-animal sources than before and although calf rennet is still used there is a reduced call in Europe for ox rennet.

Vells and Reeds are kept separate at all stages of production. This is because the two important enzymes in rennet, pepsin and chymosin, differ reciprocally in their concentrations in vells and reeds. Thus producing two kinds of rennet (from vells and from reeds) enables final products to be generated according to customer requirements for enzyme ratios. However, it was customary in GB to produce four basic types of rennet that suited most customers. These are:

- Standard cheese rennet - 85% calf 15% ox rennet

- Essence of rennet – 100% dilute calf rennet

- Stabo cheese rennet – 100% ox rennet

- Cabo cheese rennet – 50% calf and 50% ox rennet

The approximate composition of calf and ox rennet is as follows:

ANIMAL SOURCE	CHYMOSIN	PEPSIN	RENNET TYPE
Newborn calf	100%	0%	Calf
Veal calf	80-90%	10-20%	Calf
Adult bovine (ox)	20%	80%	Bovine (Ox rennet)

The age of source animals currently used for rennet production is not known but the chymosin:pepsin ratio in rennet is maintained above 75:25 (EMEA 2002) therefore a proportion of older cattle are likely to be used with the majority being six months of age or less.

Chymosin exists in two forms, chymosin A and chymosin B that are determined genetically. They differ chemically by one amino acid at position 244 (not counting the 'pro' sequence) of the appropriate gene. Thus an individual animal produces either chymosin A, or chymosin B.

Chymosin A has aspartic acid and chymosin B has glycine at position 244. This results in slight differences in electric charges and thus the isoelectric point. The two types also appear to differ slightly in their stability during storage. Commercially produced rennet is a mixture of these two chymosins. In practical terms there are no other significant differences between them and they act identically.

In summary, rennet is an essential clotting agent used during the manufacture of many cheeses. Its main function is to coagulate milk proteins such as casein. After use for this purpose rennet ends up partly in the curds (from which cheese is made) and partly in the whey that is used in animal feed and possibly infant food. Further treatment of whey enables by-products such as

lactose to be extracted. Lactose is used in medicines and confectionery. Rennet can be produced from animals, mostly calves and adult cattle, from plant materials and by genetic engineering[3]. The following report considers only rennet produced from animals and particularly calves and adult cattle. Animal-derived materials are, so far as is known, not used in the production of rennet from other sources and will not be considered further.

II.2. RISK ASSESSMENT

II.2.1. The hazard

In regard to this risk assessment, the hazard under consideration is the BSE agent. This is because the BSE agent is regarded conclusively as the cause of BSE in cattle and the probable cause of vCJD in man and TSE in various other species of *Bovidae* and *Felidae*. In the context of rennet the hazard could only stem from the abomasum of cattle because this is the only animal-derived material currently known to be used in the production of animal rennet.

II.2.2. The risk (cattle)

Because calf and ox rennet is only produced from the abomasum, the risk analysis focuses on the risk from inherent infectivity in this organ or from contaminating sources of infected material from the same or different carcases.

Since only bovine abomasums are known to be used significantly in rennet manufacture the risk analysis will consider the risk from abomasums from calves and adult cattle. Among farmed animals including those other species which are a source of rennet, only cattle are known to develop natural disease following exposure to the BSE agent in feed. Sheep and goats, but probably not pigs and poultry, are susceptible to the BSE agent in brain material experimentally administered by the oral route. However, it is pointed out that any species fed concentrate rations that might contain infected mammalian meat-and-bone-meal (MBM) or which accidentally might be come contaminated with it, could theoretically contain such infected material within the lumen of the abomasum at slaughter, whether or not there was any absorption of infectivity into the mucosal lining of the abomasum. It is noted that the feeding of a range of animal protein products, including MBM to food animal species is prohibited in the EU. Assuming perfectly enforced TSE regulations within the EU, there still could be a TSE risk from imported live calves and adult cattle, abomasums, rennet or anything containing or exposed to rennet including cheese. Any risks there may be would be dependent on the risks in source countries and particularly those of geographical BSE risk (GBR) higher than 1. Because the risk from BSE is dynamic so should be the GBR, so changes in the BSE risk for any country could alter its current classification.

In summary the ultimate or residual risk in rennet is determined by the BSE-infectivity naturally present in the abomasum or which accidentally contaminates the organ, the effect of the physical and chemical processes used

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[3] Genetic engineering was the predominantly used method in Great Britain in the year 2002.

during the production of rennet and any further reduction that may result during the manufacture of cheese from curds or further processing of the whey.

II.2.3. Geographical source of animals (cattle)

The geographical source of reeds and vells is likely to vary over time depending on the price, availability and the effects of epidemic disease like foot and mouth disease and blue tongue in countries of origin.

In the EU calf and ox rennet is currently produced from abomasums of calves and adult cattle derived from Australia and New Zealand (GBR 1), continental Europe (GBR 2 or 3) and the USA (GBR 2), (EMEA 2002). Currently, calf and ox rennet produced in the EU is used in the EU and some may also be exported. Cheese, and other by-products from whey, could be similarly used or exported directly or in products.

Currently (2002) calf and ox rennet is not produced in the UK, Ireland or Portugal (EMEA 2002). Furthermore, one company that produces commercial quantities of rennet from abomasums of cattle in Europe, claims not to source raw materials from countries with a geographical BSE risk assessment of category IV (currently the UK and Portugal), neither do they source from Switzerland or Ireland.

Historically in Great Britain approximately 90% of vells were imported from Australia, Belgium, France, New Zealand and Switzerland. The remaining ten percent were derived mostly from the West of England. Reeds were sourced 50/50 from the UK (mainly Scotland and Northern Ireland) and from external sources mostly Germany, Netherlands and the USA. Historically GB-produced calf and ox rennet was used in GB and also exported to Europe and the USA.

It is noted that some 500,000 UK born calves were exported annually from the UK to other member States mostly for veal production until 1996. The rules governing these exports demanded that no calf was the offspring of a BSE-affected dam and it had to be killed in the country of destination before it was six months old. Under these conditions any risks from BSE were regarded as negligible though even this trade was prohibited from 1996 in regard to the UK and subsequently for calves and adult cattle from Portugal.

II.2.4. Definition of source animals (cattle)

Abomasums are only sourced from animals destined for human consumption, slaughtered in licensed abattoirs following official *ante* and *post mortem* examination and passed fit for human consumption. The whole process of slaughter and subsequent procedures in the abattoir are subject to official control in accordance with Council Directives 64/433 and 93/43 and employing hazard analysis and critical control point procedures (EMEA, 2002).

II.2.5. Risks from cross contamination in the abattoir (cattle)

Embolic spread of infected material *(See EC, 2002a)*

The first risk stems from the possibility of cross-contamination as a result of the stunning procedure applied by law before killing by bleeding out. Currently electrical stunning, which is often used for calves up to six months old, is regarded as presenting a negligible risk. Older cattle, and sometimes calves, may be stunned by methods that penetrate the skull and damage the brain. Although there is no formal evidence that the method using a cartridge operated captive bolt pistol can cause brain emboli to enter the venous system, more research has been advised to confirm this view. Captive bolt pistols that inject air under pressure into the cranial cavity, and any brain penetrating method followed by pithing has been shown in some cases to create risks of brain emboli being discharged into the venous system. Such emboli are likely to be trapped in the right side of the heart or the lungs or be present in the blood that exits from the stick wound to the blood trough. However, a recent report on these topics (EC, 2002a) considers that currently available data on bovine stunning are not conclusive and recommends that further work be done to verify this and to investigate the risk of emboli getting to the systemic circulation and thus be distributed to remote tissues that could include the abomasum. Current methods of stunning that do present a risk from emboli are banned for use throughout the EU. Furthermore, as a result of the use of compulsory PrP testing of brains from animals over 30 months old throughout the EU (and over 24 months if animals are for casualty slaughter) and the removal and destruction of the carcass and organs of positives and the carcass and organs from the animal immediately preceding and two carcases following the positive on the slaughter line, any residual risks are minimal. Thus, if current laws are followed and enforced, the risk of BSE infection being disseminated to the abomasum by embolic spread from the central nervous system in the EU, are regarded as negligible (EC, 2002a).

Risks from accidental cross-contamination from specified risk materials (SRM)

SRM from calves and adult cattle are the tissues that in slaughter cattle might potentially harbour most of the BSE infection. The risks from brain have been partly described in the paragraph above. The possible risk from blood (other than within the circulatory system – see above) are negligible because bleeding is done with the carcass in the vertical, hanging position at a point below the abdomen which is not opened until bleeding has ceased. Because the head is removed before the abdomen is opened there is no risk of cross-contaminating abomasum by direct or indirect contact if hygiene rules are followed. There is no risk either from spinal cord or any part of the vertebral column since the carcass is not split to expose these structures until after the stomachs and intestine are removed from the abdominal cavity and they enter the gut room. Rodding (closure and sealing of the oesophagus to prevent leakage of stomach contents) and bagging (sealing of the rectum to prevent faecal leakage) prevent any contamination of the external surface of the abomasum from these sources.

On entry to the gut room the intestine is removed from the stomachs by severing the duodenum at the pylorus within about 1 cm of the pyloric sphincter. The intestines are collectively SRM, but in experimentally BSE-infected animals, infectivity has only been reported in the distal ileum, several metres distant from the severance point. Thus the risk from this source can be regarded as negligible.

The abomasum is separated from the omasum by cutting. In limited studies (see next section for more details), BSE infectivity has not been detected in the omasum or any of the fore-stomachs in natural or experimental BSE thus any BSE-risk by cross contamination from these tissues can be regarded as negligible.

The contents of the abomasum are expelled by squeezing from the omasal, to the pyloric (duodenal) end. The abomasum is hung and external fat and adherent tissues (that might contain haemal nodes and/or small lymph nodes or haemal lymph nodes) are removed. BSE infectivity has not been found in lymph nodes or midrum (mesenteric) fat, thus contamination from these sources can be regarded as negligible especially as they are removed without cutting through the capsule of the nodes if present. In fact this is potentially a risk reduction procedure because if any minimal infectivity were rarely present, it would be removed before onward processing.

Vells are not opened or washed. They are frozen in batches of 24 or 25 (100 if from Australia) for despatch and retained frozen at below -15ºC before use.

Reeds are similarly de-fatted, trimmed, cut open and the mucosal surface rinsed lightly with water. The neck of the pyloric part is removed. The remainder is frozen in blocks of 20 on the day of the kill, for despatch to the processing laboratory. They are retained frozen at below -15ºC before use.

II.2.6. Intrinsic risks from BSE infectivity within the bovine abomasum (cattle)

There are theoretically two main tissues within the wall of the abomasum that could harbour or replicate BSE infectivity. These are nervous tissue and lymphoreticular tissue. No-one has yet devised a method to show that the myenteric plexuses of the gut specifically contain infectivity but research in mice (Kimberlin, 1986) and hamsters (Beekes, Baldauf and Diringer; 1996, McBride and Beekes, 1999) has shown that the autonomic nervous system is involved in the transport of infectivity between gut and central nervous system. In the newborn calf organised lymphoreticular tissue within the wall of the abomasum is absent. Older calves have low numbers of lymphoid follicles present. There are larger numbers of lymphoid nodules in the *lamina propria* of the abomasum from adult animals. Some of these have prominent germinal centres and the nodules sometimes penetrate into the sub-mucosa. There are also large numbers of cells of the lymphocyte series in the superficial layers of the mucosa. These are more numerous in reeds than in vells (R. Bradley, unpublished).

Theoretically therefore**,** there are tissues in the abomasum of calves and adult cattle that can harbour TSE infection and permit TSE agent replication. Because these tissues are distributed as intrinsic components of the wall of the abomasum and therefore cannot, for all practical purposes, be dissected, they

cannot be bioassayed individually for infectivity. However, using PrP^{Sc} as a marker of infectivity, immunohistochemical examination of the abomasum could inform on their disease status, but there are no reports of such studies specifically on the abomasum of calves or adult cattle exposed to, or infected with, the BSE agent.

Some indirect evidence of the unlikely occurrence of infectivity in any tissue component of the abomasum in cattle infected with BSE comes from the collective bioassays that have been done on bovine tissues from natural and experimental BSE, indicating a more restricted distribution of infectivity than occurs in natural or experimental TSE of sheep (EC, 2002). Recently, immunohistochemical studies of the presence of PrP^{Sc} in the intestines of natural cases of BSE and experimentally exposed calves have provided data which complements results of previous infectivity studies.

Studies of infectivity and/or PrP^{Sc} in the stomachs and intestines of cattle with naturally occurring BSE or after experimental exposure to brain tissue from natural, confirmed cases of BSE, are summarised as follows:

Natural cases of BSE: No infectivity was found, by assay in RIII mice (inoculated by the i/c and i/p routes), of the following individual tissues from clinical cases of BSE: epithelium of rumen, reticulum, omasum, abomasum, distal ileum and proximal colon (from one case), epithelium of proximal intestine, distal colon and rectum (from another case) and distal small intestine (from a third case) (Fraser and Foster, 1994). In an immunohistochemical study of PrP^{Sc} in the distal ileum (but no other parts of the alimentary tract) (Terry et al. submitted for publication) no immuno-staining was detected in the lymphoid tissue of distal ileum of 29 naturally occurring clinical cases of BSE. In 9 of these cattle, small areas of fine granular staining were observed in the myenteric plexus.

Experimental BSE: Infectivity, assayed (by mouse inoculation) in the wall of the pyloric region of the abomasum has not been detected in cattle killed sequentially throughout the incubation period after experimental oral exposure to brain tissue from natural, confirmed cases of BSE (Wells et al, 1996; Wells et al, 1998; EC, 2002). Furthermore, infectivity in a comprehensive range of alimentary tissues (Wells et al, 1996) from animals of this study was confined to the distal ileum (Wells et al, 1998; EC, 2002). In an immunohistochemical study of PrP^{Sc} in the distal ileum from animals of the same study, the presence of the disease specific form of the prion protein in lymphoid tissue, according to sequential time points throughout the incubation period concurred with the previous infectivity findings (Terry et al. 2002, submitted for publication). Also, PrP^{Sc} in the nerve plexuses was found only in the myenteric plexus, and then, only after the onset of clinical signs in one animal killed at 38 months after exposure and another one killed at 40 months after exposure. In an additional immunohistochemical study mesenteric lymph nodes and all levels of the intestine (but not abomasum) were examined for PrP^{Sc}. In this study, three 4-6 month old calves were exposed orally to 100g brain from natural cases of BSE and killed at 10-12 months of age. PrP^{Sc} detection was confined to the lymphoid tissue of the distal ileum (Wells, 2001; Terry et al. 2002,

submitted for publication). No immuno-staining was detected in either the myenteric or the sub-mucosal plexuses of the intestines from these animals.

This is clearly in contrast to sheep with natural scrapie, in which widespread involvement of both lymphoid tissues and enteric nervous system neurones have been described from early in the incubation period. BSE risks from this source in calves and adult cattle are therefore unlikely.

This does not mean it is devoid of risk. To overcome the reduced sensitivity of mice over cattle to detect BSE infectivity in cattle tissues, a number of tissues from experimentally BSE-challenged cattle have been inoculated into cattle by the i/c route. None of the tissues that showed no detectable infectivity in mice have so far shown infectivity when inoculated into cattle. However, the abomasum has not been inoculated in this way.

Overall these studies show conclusively that the distribution of BSE infectivity in cattle with natural or experimental BSE is more restricted when compared with the distribution of scrapie infectivity in sheep or goats with natural or experimental scrapie. In fact, in calves and adult cattle the only alimentary tissue in which BSE infectivity has been found is the distal ileum, which is several metres distal to the abomasum and thus, if appropriate meat hygiene rules are enforced, there is a negligible risk of contaminating the abomasum with the distal ileum.

II.2.7. The potential problem of infected feed (cattle)

From the above it could be concluded that there is no evidence for there being a high level of BSE infectivity in the abomasum of healthy calves and adult cattle. Indeed all the evidence available indicates such inherent infection is either absent, or present at such a low level that it cannot be detected by the mouse bioassay.

If BSE-infected MBM was in the diet of calves and adult cattle, whether infected with BSE or not, it is theoretically possible that this MBM could be present in the lumen of the abomasum and other parts of the gut at the time of slaughter. Thus, the mucosal surface of the abomasum could be contaminated with an external source of BSE infection. Since 1994 throughout the EU the inclusion of MBM in the diet of ruminant animals has been prohibited and from 2000 it has not been permitted in the diet of any food animal species. Although there have been some breaches of this legislation in several countries, their frequency in 2002 is regarded as being very low. Furthermore, effective removal of SRM and improved standards of rendering make the risk from MBM much lower than previously. In any event, it would be most unlikely that abomasums of newborn calves could be contaminated in this way because they would not consume significant levels of solid feed. If BSE-infected MBM nevertheless accidentally were introduced into calves and adult cattle rations, adult cattle would be at greatest risk as they are more likely to consume solid feed of this kind. Whereas the risk from infected MBM should not be completely ignored, it is anticipated to be a very low risk in 2002 if the recommendations made in the various SSC opinions with respect to TSE risks are well enforced.

The abomasums from newborn and veal calves up to six months old would be at greater risk than adults from infected feed in the lumen following the feeding of milk substitutes if these contained BSE-infected material. Possible sources could be bovine fat not from discrete adipose tissue (though infectivity has not been demonstrated therein) or fat derived from the degreasing of bones (especially vertebral and skull bones) used for the production of bovine bone gelatine. Skulls of cattle above 12 months are now SRM in the EU and vertebral bones from cattle over a year old are not permitted into any food chain (with certain safe exceptions because comparable measures are in place, *e.g.* in the UK, the Over Thirty Months Scheme). Only fat from discrete adipose tissues without additional treatment (such as pressure cooking) is recommended to be used in animal feeds in the EU (SSC Opinion, 2001), therefore if this opinion is converted into legislation, risks from contamination of the lumen of the abomasum with BSE-infected feed is remote and hypothetical. The risks can in any case currently be considered as negligible provided all the current appropriate legislation (such as SRM legislation) is enforced. [NOTE: should the SSC Opinion be converted into legislation this section should be re-visited and amended if appropriate. The section should be similarly re-visited and possibly updated/amended later in the light of the forthcoming quantitative risk assessment of milk replacers].

II.2.8. Processing of vells and reeds (cattle)

The method of production of calf and ox rennet is commercially restricted information. However, the principal features of the process used in the UK in 1989 involved mincing, extracting, pressing, acidification and clarifying, filtering, concentrating, further filtration, standardising, microbiological testing, strength testing, packaging, storage and despatch. Rennet can also be produced in lyophilised form (EMEA, 2002) from the liquid end-product.

A generic description and principal features of current methodology provided by the industry on 18 April 2002 is as follows:

Mincing frozen (or sometimes dried) stomachs, extraction of the enzymes, mechanical separation of extract from stomach residues, activation of the pro-enzymes at acid pH (pH 2.0), neutralisation (pH 5.5) and clarification of the extract (by filtration or centrifugation), concentration *e.g.* by ultrafiltration, formulation, standardisation, sterile filtration and quality control, (Harboe and Budtz, 1999). Some products are purified further by ion exchange chromatography mainly to obtain a product with a higher percentage of chymosin than that naturally present in the stomach ((Harboe and Budtz, 1999).

- Extraction of the minced stomachs takes place in water, with or without added salt, buffer and/or preservatives.

- Mechanical separation may take place by centrifugation or filtration.

- Activation of the pro-enzymes involves pH 2 or slightly higher, by addition of strong acids. This cleaves the pro-enzyme, leaving the active, mature enzyme and a shorter, inactive pro-peptide sequence. Many other inactive proteins will precipitate during this step. This is followed by neutralization by addition of base to about pH 5.5 after at least half an hour, without heating, *i.e.* it takes place at ambient temperature.

- Clarifying may require addition of flocculants followed by filtration (one or more steps) or centrifugation. Filter aid may be used.

- Concentration, if needed, may take place by ultra-filtration.

- There may also be a chromatographic step (ion exchange) if it is necessary to separate pepsin and chymosin, for production of rennet particularly high in chymosin. It is noted that that there is currently less call in Europe for calf rennet with an extremely high chymosin content.

- Formulation involves addition of salt, preservative (normally sodium benzoate), buffer and for some markets monopropylene glycol.

- Sterile filtration is through a fine filter, *e.g.* 0.2 micrometer absolute filter.

Effectiveness of Processing in reducing any TSE infectivity:

Enzymes, including milk-coagulating enzymes, are delicate substances, which do not tolerate physical or chemical treatments that are known to be effective in reducing TSE infectivity titres. Complete destruction of any TSE agent that might unexpectedly be present in the starting material is therefore unlikely. However, processes are used in the manufacturing process that might remove contaminating prions, particularly if they existed in aggregated form, or were present within, or attached to cells. Sequential filtrations would be one such procedure that might contribute to prion removal.

There are no available data on what infectivity reductions might occur in the production process as a whole (*e.g.* as a result of spiking studies). In this regard any TSE infectivity in the final product would result from infectivity in the starting material. Since in 2002 that can be regarded as negligible, any risk in the rennet can also be regarded as negligible.

Summary:

There is no individual step in the process that is likely to guarantee destruction of any TSE agent present. However, there are processes such as acid treatment and particularly filtration sequences that might contribute to TSE agent removal/inactivation. Quantification of any reduction that may occur is currently not possible.

II.2.9. Potential for improved TSE risk reduction during processing (cattle)

Experiments involving acid inactivation of hamster scrapie prion rods (Appel, Groschup and Riesner, 1999) showed that 1N hydrochloric acid at 65°C or above for 1 hour led to almost complete inactivation. The method of acidification used in rennet manufacture is not published and it is not known if either the acidity or temperature would be detrimental to the enzymes present in rennet. However, it might be possible to introduce this process or a similar one that could give a sound assurance of risk reducing powers of the process.

Tateishi *et al* (2001), have reported high infectivity reduction rates of scrapie infectivity by the use of Planova virus removal filters with a pore size of 15nm. The nature and detail of the filtration processes used in rennet manufacture are not published but consideration of the work reported here may assist in providing additional assurance as to the capability of current

filtration methods, or the addition or substitution with Planova filters or others giving equivalent reduction rates.

Meyer *et al* (2000) report on the use of high pressure for the sterilisation of foods. Though the effect of pressure alone on rennet and on TSE agents is as yet unclear, there is certainly evidence that high-pressure (during rendering using pressure cooking for example and in autoclaving) could have a useful TSE infectivity reducing effect whilst being non-damaging to the enzymes.

II.2.10. Presence of cells in rennet (cattle)

Rennet is a cell free end-product and is microbiologically sterile, at least in regard to bacteria. The pore size of the filters used, ensures that whole cells cannot be present. Thus no lymphatic cells are present in rennet. It cannot be excluded that cellular organelles small enough to pass through the pores of filters could be present but there is no evidence for this or that this presents a risk.

II.2.11. TSE risks in animal rennet prepared from the abomasum/stomach of species other than cattle

Of food animal species, none other than cattle naturally contract BSE. Thus any BSE risk can currently be regarded as negligible except as the result of contamination by BSE-infected feed that is in the stomach at the time of slaughter (*e.g.* as a result of importation of imported MBM or feed containing MBM from a BSE-infected country). It follows therefore that rennet produced from the stomachs of sheep, goats, pigs and hares has a similar negligible BSE risk to calf and ox rennet if this risk is avoided.

Sheep and goats may be naturally infected with scrapie, a TSE without a known risk for man. There is less information available on the scrapie infectivity of different parts of the alimentary tract including the abomasum of these species than there is in cattle. However, there is known to be a wider tissue distribution of scrapie infectivity in sheep and goats than of BSE infectivity in cattle. Furthermore, PrP^{Sc} has been reported to be widely distributed in the nervous tissue and organised lymphoid tissue of the alimentary tract of sheep with natural scrapie (van Keulen *et al* 1999, Andreoletti *et al* 2000). Indeed, the Scientific Opinion on TSE Infectivity distribution in ruminant tissues (state of knowledge, December 2001) (EC 2002b) reports that sheep with the *PrP* genotype ARQ/ARQ when experimentally infected with BSE show the presence of PrP^{Sc} in the abomasum both in the pre-clinical and clinical stage of disease. However no PrP^{Sc} is found in the abomasum of ARR/ARR or ARQ/ARR sheep. Based on these continuing experimental BSE studies in sheep it seems likely that if sheep and goats become naturally infected with BSE a similar distribution as in natural scrapie might ensue. The distribution of scrapie infectivity is influenced by the *PrP* genotype of the host (van Keulen *et al* 1999). Though studies in goats have not been done and reports of scrapie in this species are generally less frequent than in sheep, the same general principles apply. (Note an exception for iatrogenic scrapie in goats in Italy resultant upon the use of contaminated locally-produced vaccines, (Agrimi *et al,* 1999; Capucchio *et al,* 1998; Caramelli *et al,* 2001)). Since it appears likely that scrapie infectivity could be

present in the abomasum of sheep and goats with scrapie, it cannot be excluded that some of this infectivity could be present in rennet. On the basis of current knowledge it is unreliable to depend on the processing to remove it, though the titre of infectivity may be reduced.

II.2.12. Dependence on the country source of starting materials for rennet safety

In regard to calves and adult cattle, it is an essential risk management procedure to avoid the feeding of ruminant animals with mammalian protein and specifically ruminant protein. If this is effectively carried out, then the TSE risk from feed in the abomasum at slaughter is negligible. If this feed ban is applied to all food-producing animals in the country under consideration all these species are similarly protected from this source.

In regard to bovine abomasums collected under the same or equivalent conditions as in the EU then any BSE risk in calf and ox rennet can be considered negligible.

The ultimate test for bovine abomasums is the GBR category of the source country in question (which should be regularly reviewed). Category 1 countries present a negligible risk. Other countries should be assessed on their merits because the following factors could influence the safety of rennet: the method of stunning, carcass dressing and abomasum collection.

II.2.13. Summary on the TSE risk in rennet

According to the best information available from the industry and the Report (EMEA, 2002), rennet can be produced from solely plant material, can be genetically engineered in which case there can be no BSE or TSE risk as the hazard is definitively absent, or can be produced from the stomach of animals in which case a TSE risk is theoretically possible. In practice, commercial quantities of calf and ox rennet are only produced from the abomasum of calves and adult cattle; no other animal derived material is used in its manufacture. Thus, any risk there may be in calf and ox rennet stems only from any possible inherent BSE infectivity present within the organ during life or any cross-contamination from a BSE-infected source. In regard to the former, nervous and organised lymphatic tissue occur in the abomasal wall and such tissues in other sites and species can harbour PrP^{Sc} and/or replicate TSE infectivity, but no BSE infectivity has been found in the abomasum of calves and adult cattle. In regard to cross contamination the only possible source could be from SRM since no animal tissue or products are brought into contact with any raw materials used in the manufacture of rennet after abomasums leave the abattoir.

In theory SRM could contaminate the abomasum in three possible ways:

- As a result of the stunning process: see the Opinion of 10-11 January 2002 of the SSC on Stunning methods and BSE risks (EC, 2002a).

- From cross-contamination by SRM (but current meat hygiene regulations and methods of carcass dressing, if enforced, make it impossible for the abomasum to be contacted with the infected surfaces of SRM), or

- From BSE-infected feed in the stomach at the time of slaughter (but mammalian MBM and certain fat tissues in calve and adult cattle diets that theoretically might contain BSE infectivity are now prohibited in the feed of all food animal species).

Although the processing applied to collected abomasums for the production of calf and ox rennet has not been tested to show the extent of titre reduction, it is likely that any TSE-infectivity present in the starting material is reduced, notably by the filtration methods employed. However, there is no discrete part of the processing that is likely to entirely eliminate any infectivity present nor is this likely from the collective effect of all treatments.

It follows that any material produced downstream from the point of addition of rennet (such as cheese, whey, lactose) will have no measurable increased risk of TSE infectivity as a result of the addition of rennet, so long as no other animal-derived materials are added or used. If they are, then a further risk assessment is required for each animal product added. In practice the starting materials for the production of curds and whey are milk and rennet. Bovine milk has already been accepted internationally as a safe end-product from healthy cows without clinical signs of BSE (EC, 2001). BSE has not been reported in buffalos whose milk can also be used for human consumption in the EU. It is noted that leukocytes (including lymphocytes) exist naturally in ruminant milk and their frequency is controlled by regulation and testing.

III. CONCLUSIONS

At the present time, BSE risks are only possible from calves and adult cattle since this is the only food animal species naturally affected by BSE. BSE risks from small ruminants are hypothetical and speculative at the present time and the risk assessment for this is reported elsewhere. TSE infectivity in rennet from scrapie-infected small ruminants is theoretically possible but currently is not believed to create a danger for humans. The source of any infectivity in rennet is only from the abomasum or from TSE-infected feed within its lumen at the time of slaughter. A number of TSE-risk reduction strategies are currently in use in abattoirs in the EU that reduce risks from cross-contamination and should eliminate them if properly enforced. Currently, bioassays have not demonstrated BSE infectivity in calf and adult cattle abomasum but any future studies using cattle or transgenic bovine mice should be taken into account in reassessing any risk. The situation in small ruminant abomasums is less certain other than the fact that BSE has never been reported to naturally occur in these species.

III.1. CALF AND OX RENNET

Source

Under the conditions of collection and storage specified in the above report, which imply feed-bans and avoidance of cross-contamination, and in the light of current knowledge, a BSE risk in rennet derived from the abomasums of calves and adult cattle of any age slaughtered and passed fit for human consumption in the EU is negligible. The same negligible risk is present in the abomasums from calves and adult cattle in GBR category 1 countries.

Process

The precise details of processing methods used historically and currently are commercially sensitive and commercial in confidence. No spiking studies have been reported that can effectively inform on the ability of any single part or of the whole process to destroy or eliminate any TSE infectivity that may unexpectedly be present. Enzymes, including those present in rennet, are sensitive to the harsh treatment currently needed to ensure the destruction of TSE infectivity and so are contra-indicated. Nevertheless, some treatments used during processing like acidification with strong acid to pH 2.0 might have a small, but unknown effect. In addition there are some features of the processing, of which filtration is perhaps the most likely, that may contribute to TSE agent removal particularly if it were aggregated or within cells or attached to cells. However, no quantification of the effectiveness of this process is available.

Use

The rennet to milk ratio in use is in the order of 1:10,000. Thus any TSE infectivity in the final rennet product would need to be extremely high (which seems unlikely considering the large number of abomasums used to make rennet and the other features of sourcing indicated above). Furthermore, although not calculated, the residual infectivity in any rennet still present in cheese, whey or other by-product would also have to be high to produce an effective oral dose across a species barrier which also seems unlikely as relatively small amounts of these commodities are consumed at one sitting.

Rennet is mainly used in cheese making and some may be used in cooking. Other by-products of bovine milk following treatment with rennet, such as lactose, have a wide variety of uses including in medicinal products. Alternative, non-animal sources of calf and ox rennet are available that avoid all risks from TSE agents but may not be preferred in all circumstances.

Conclusions:

The TSE safety of calf and ox rennet is largely, if not entirely, determined by the safety of the source material. Certainly safe sourcing is a key factor and is possible. Currently in the EU, any TSE risks in the source material could be considered negligible. Processing is unlikely to have significant TSE agent inactivating capacity but may be able to remove at least some aggregated infectious material if it were unexpectedly present. Alternative forms of rennet that avoid any TSE risk are available, but may not be suitable for all purposes or may not be preferred.

III.2. RENNET FROM OTHER SPECIES

Provided that BSE-infected feed is not present in the alimentary tract of **pigs** at slaughter any risk in rennet prepared in a similar manner is negligible.

Should it be probable that BSE is present in small ruminant flocks under field conditions, then the rennet may represent a possible risk because of the recently reported presence of the BSE agent in the abomasum and forestomach of sheep experimentally infected with BSE (Jeffrey, 2001). The rennet

prepared from these species by a similar method is therefore negligible only provided (1) that the presence of BSE-infected feed in the alimentary tract of small ruminants (sheep and goats) is excluded (e.g., because of feed-bans) and (2) provided TSEs in small ruminants has been proven not to occur in the country of origin and that small ruminants in the past have never otherwise been exposed to BSE infectivity. Alternatively, the flocks should have been certified as representing a negligible risk on the basis of the criteria established in the report attached to the SSC opinion on safe sourcing of small ruminant materials adopted on 4-5 April 2002 (EC, 2002c). Given the current unknowns with regard to BSE in sheep, the TSE/BSE *ad hoc* Group recommends that for the time being, and until surveillance data have provided a clear picture of the incidence of TSEs in small ruminants and on the possible presence of BSE, that rennet for pharmaceutical applications (including for the production of pharmaceutical lactose) is not derived from small ruminants.

III.3. RENNET FROM NON-ANIMAL SOURCES

In the UK and probably in the rest of the EU the majority of rennet used is from non-animal sources that present no risk from TSE so long as no other animal derived material is added.

III.4 DERIVATIVES FROM RENNET-TREATED MILK

It follows from the above that derivatives from rennet-treated milk as defined in this report, including cheese derived from curds and lactose lactulose, galactose and ethanol derived from whey all have a similar negligible BSE risk, provided no other animal-derived product is used during their manufacture. It is noted that currently there are a number of alternative sources than animals (including cattle) for rennet production that could eliminate any TSE risk.

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