Strategies and methods to detect and quantify mammalian tissues in feedstuffs: *A summary of the EU STRATFEED project (G6RD-2000-CT-00414).*

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Introduction

The UK BSE/vCJD and FMD epidemics were catastrophic failures in animal feed hygiene that caused great harm to the livestock industry and great loss in consumer confidence at home and abroad (Phillips *et al, 2000,* Pennington, 2003, Ridley and Baker 1998, Rhodes, 1998, North, 2001). As a result we can expect greater surveillance and traceability imposed on the feed industry in future (ACAF, 2003, 2004). It is a characteristic of EU legislation that it is imposed with the expectation that appropriate technical methods will be devised in due course to meet the needs of compliance with the law. Resulting from the ban on the use of meat and bone meal (MBM) in animal feeds (94/381/EC; 2000/766/EC), the EU implemented the three year STRATFEED project to research "Strategies and methods to detect and quantify mammalian tissues in feedingstuffs". The study involved ten European partners who collaborated in developing and testing four different methods to detect MBM in feeds:

1. Optical Microscopy OM (the official method)
2. Near Infrared Spectroscopy, NIRS
3. Near Infrared Micro-spectroscopy, NIRM (and NIR Camera)
4. Molecular biology methods: Polymerase Chain reaction, PCR

This paper summarises the outcome of the STRATFEED project which was publicised in its recent International Symposium: "Food and feed safety in the context of prion diseases." Held in Namur, Belgium 16-18 June 2004 hosted by CRA-W, JRC-IRMM, AFSCA and Agrobiopole. Full details of the symposium including presentations can be found on the very informative STRATFEED website (Vermuelen *et al,* 2003): http://stratfeed.cra.wallonie.be/

Suffice to say that no single method fulfils all the ideals of universal applicability, reliability, throughput, limit of detection (LOD) and, not least, the cost. However the methods do show complementarity forming the basis for a control strategy.

Background

It is not certain what initiated the BSE outbreak in the UK cattle herd (Phillips *et al*, 2000, Horn 2001, Asante *et al,*2002). The introduction of milk quotas in 1984 may have led to dairy farmers attempting to maximise milk yield in the year before by increasing the protein in the diet and MBM was a cheap protein source at that time. Changes in the rendering process concurred with this time and may have contributed to transmission of infectivity. Infectivity is believed to be caused by conformational changes in prion proteins from normal alpha helix (PrPC) to beta pleated sheets (PrPSc) which recruit normal proteins to join their cause as rogue prions.(Prusner1991, 1995, 1997)

Because of the long incubation time of TSE's and the unrecognized nature of the disease, it was some considerable time before the outbreak was realized, by which time a sufficiently large cohort of animals were incubating BSE and contributing to its propagation from recycling their carcass slaughter wastes as MBM. In spite of the established wisdom of taboos forbidding cannibalism and the research work of Carlton Gadjusek (1996) on cannibalism causing the TSE Kuru in the Fore Indians of Papua, New Guinea, cannibalism was being imposed on dairy cattle by such practices. When the first BSE cases became recognized in 1986, the disease was seen to be similar to scrapie in sheep. Scrapie had been known in sheep since the eighteenth century and there was no evidence to suggest it could cause disease in humans. Perhaps for these reasons action to contain the outbreak was initially not rigorous enough.

Only when very young people were contracting a new form of CJD (variant CJD) did concern grow and rigorous control was imposed to remove MBM and specified risk material from the food chain and exclude cattle over 30 months of age from the human diet. The decrease in incidence of BSE (Figure 1) is proof both that the measures were effective and that MBM was the most likely source of the outbreak. Due to the long incubation period infected animals and feeds spread the disease to other European countries. (Figure 2) To date just under 150 cases of variant CJD have been found in the human population in the UK (NCJDSU, 2004). The UK and EU authorities have taken action to ensure eradication of BSE. In particular the EU instigated the STRATFEED research project to devise and improve methods to detect mammalian tissue in feeds.

Although the BSE epidemic may be under control and soon eradicated, intra-species recycling (cannibalism) must be prevented in future. The present rejection, within the EU, of some 14 M tonnes per year of mammalian slaughter wastes containing 55% protein (Wolf,1982) cannot be acceptable on environmental grounds. However any subsequent reintroduction of species specific rendering and recycling will demand harmonization of high hygiene standards underpinned by robust regulatory measures. A monograph on the risk analysis of prion diseases in animals has recently been published by OIE (Lasmézas and Adams, 2003) in which the chapter by Gizzi *et al* concerns test methods.

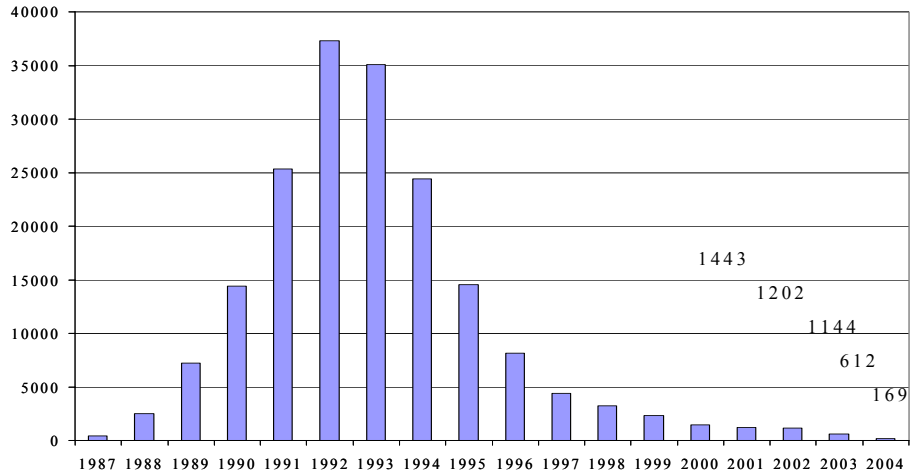


Figure 1 Reported BSE cases UK (www.oie.int/eng/info/en_esbru.htm)

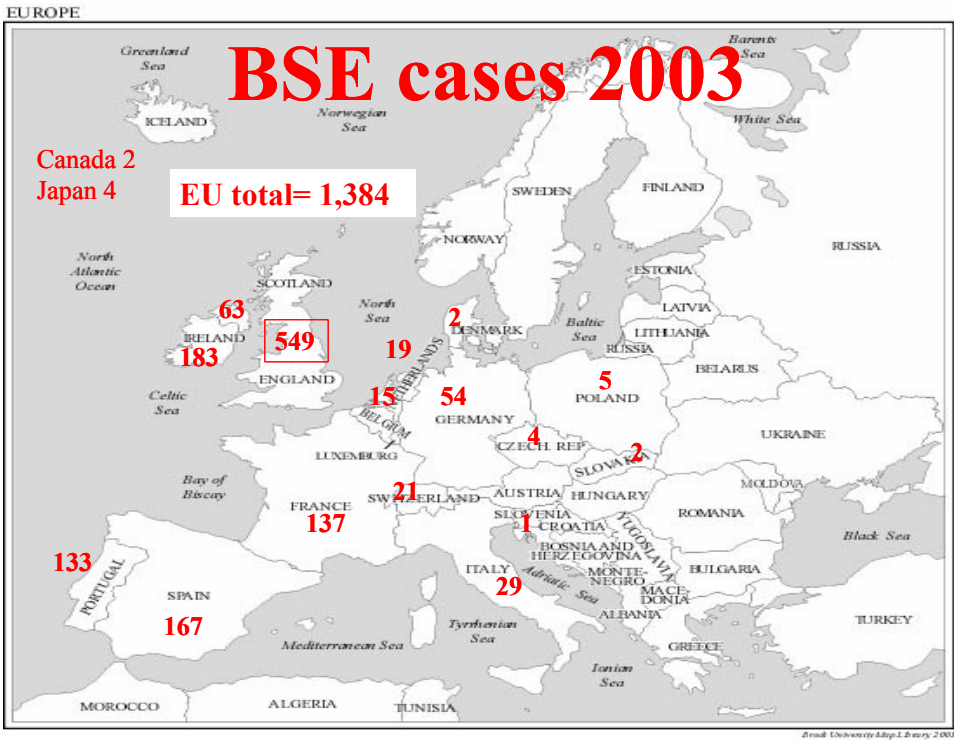


Figure 2 Reported BSE cases 2003 (www.oie.int/eng/info/en_esbru.htm)

Methods

Optical Microscopy OM

Optical microscopy of feeds remains the officially recognized method for the detection of animal tissues in feeds (Directive 88/1998/EC). However its use depends particularly on the detection of dense bone tissue which may be assigned on morphological criteria (Haversian canals and lacunae) to particular animal species by an experienced microscopist. Success of the method arises from taking a large test portion (≥ 10 g) and using tetrachloroethylene (s.g.1.62) to form a sediment containing bone (s.g.1.67-2.0) which can be examined under low and high magnification (Figure 3). If bone is present, assignment to species needs skill and experience. Stratfeed microscopists developed a micrograph gallery (Figure 4) and the ARIES (Animal Remains Identification & Evaluation System) decision support system to assist in training and harmonization of the Stratfeed protocol for OM (Directive 126/2003/EC). A limit of detection (LOD) of 0.1% MBM in feeds can be achieved. Initially the presence of fishmeal (FM) containing fish bones confounded detection but recent validation has shown this problem to be substantially improved (van Raamsdonk and van der Voet, 2003). Poultry long bones are still difficult to differentiate from mammal bone. Definitive species identification is still needed in suspect specimens for which taxon specific molecular biology methods (PCR, discussed later) are the most appropriate. Nevertheless OM is likely to remain important as a key forensic test.

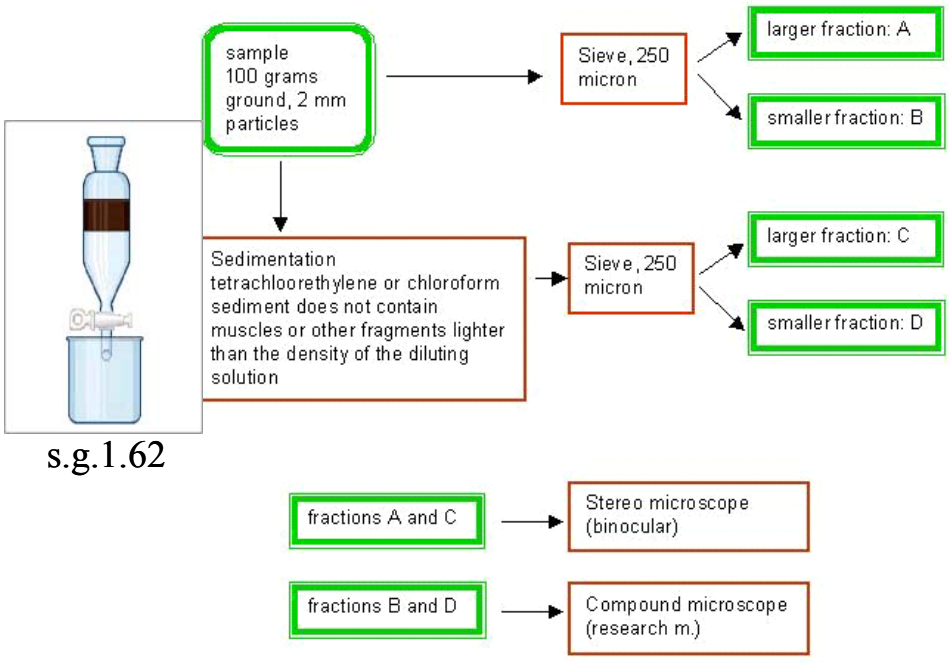
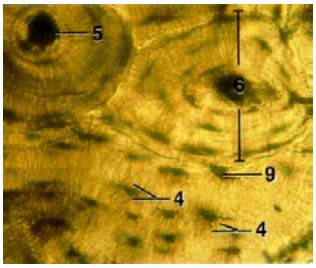
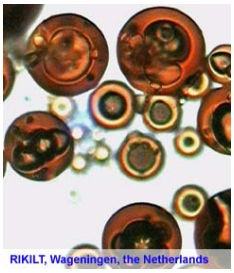


Figure 3 Protocol for Optical Microscopy of feed



MAMMAL BONE



BLOOD

MUSCLE

EGG SHELL

FISH SCALES

MAMMAL HAIR

Figure 4 Optical Microscopy of feeds. Picture gallery from ARIES decision support system (van Raamsdonk, RIKILT, NL)

Polymerase Chain Reaction PCR

Molecular biology methods hold the promise of unequivocal species identification of tissue as well as offering a low limit of detection (LOD 0.05%) even with multiple species present. The development of real-time polymerase chain reaction (RT-PCR) has transformed forensic science. It is important to choose a ruminant DNA target that is abundant in tissues; that is the DNA target has a large copy number. Mitochondrial DNA is most appropriate in this context because mitochondria are abundant in most tissues.

A mitochondrial DNA segment (271base pairs) was first used in 1998 to design a PCR test to detect ruminant tissue in feed at 0.125% MBM (Tartaglia *et al* 1998). However a 24 lab ring trial (JRC, 1999) showed the method was not fit for purpose, having over 20% false positives and over 20% false negatives. Failure was ascribed to DNA extraction and clean-up or polymerase inhibition or thermal degradation of DNA by rendering.

Evidence for thermal fragmentation of DNA showed that shorter targets (~60bp) endured higher rendering temperatures than longer targets. PCR using RFLP (Restriction Fragment Length Polymorphism) amplifies a fragment common to several species using universal primers then applying several restriction endonucleases to detect species specific internal differences. This method allowed detection of cattle, buffalo, sheep, goat, horse, pig, chicken and turkey in feeds with 0.5% processed animal protein present in a feed (Bellagamba *et al, 2001*) Because high temperature rendering is mandatory (≥ 133ºC), short DNA

targets (~60bp) must be chosen which are inappropriate for traditional agarose gel electrophoresis PCR. For this reason RT-PCR becomes the only appropriate method. Such methods use fluorescent probes in thermocyclers that are complex and elegant. (Foy and Parkes, 2001)

PCR involves melting double stranded DNA at 90ºC to form two separate complementary strands (Figure 5). This is followed by annealing of forward and reverse primers onto each strand at 37ºC followed by chain elongation at 75ºC to form two pairs of DNA molecules that subsequently repeat the cycle. Each successive thermal cycle doubles the number of copies leading to exponential amplification shown by a fluorescent signal. Primers are chosen to bracket the target DNA region characteristic of the bovine species to be detected.
The advent of real-time PCR allows visual progress of amplification (Figure 6) with each successive thermal cycle. If bovine DNA is present in the test portion taken for analysis (~100 mg), this becomes amplified after several cycles to produce an exponential appearance of the PCR product shown on the screen as a steeply rising curve. If the exponential rise occurs after, say, 15 cycles then the sample contains a considerable amount of the bovine target DNA. If the exponential rise occurs after, say, 35 cycles then the sample contains very small amounts of bovine DNA. If no exponential rise is observed at all then the specimen is free from any bovine target DNA. RT-PCR thus gives a semi-quantitative result as well as a qualitative 'Yes/No' detection. However thermal damage to DNA can reduce the signal confounding true quantitative detection.

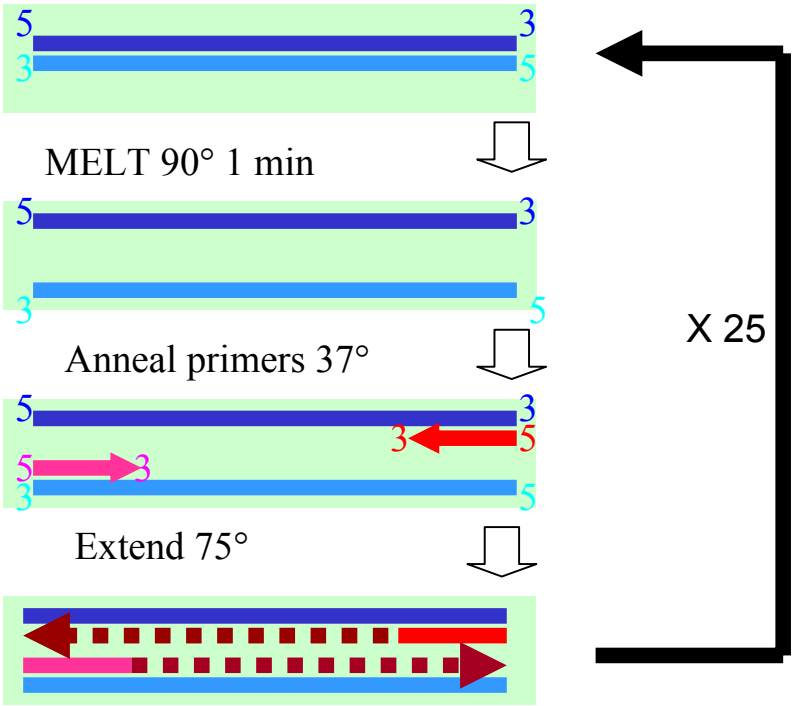


Figure 5 Outline of PCR thermo cycle to amplify DNA. Primers bracket the target.

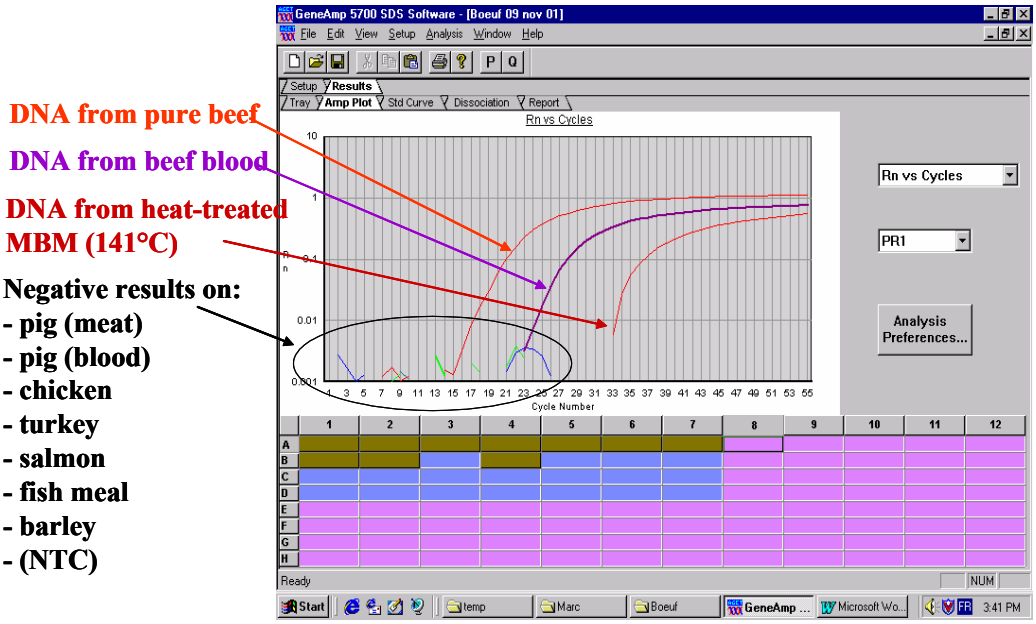


Figure 6. Real-time PCR screen showing exponential rise after 15 cycles for DNA from pure beef, 23 cycles for beef blood and 33 cycles from bovine MBM 141ºC.

Disadvantages of PCR are the small test portion taken (100mg) that risks sampling error. Furthermore milk, blood or rendered fat can yield traces of ruminant DNA that are detectable. The very high sensitivity of PCR risks accidental cross-contamination requiring dedicated labs as well as the high cost of a real-time thermocycler, reagents and skilled staff. In time PCR may well become the 'gold standard' for unequivocal species detection but its use may be restricted to well resourced regulatory labs having skilled staff.

Near Infrared Spectroscopy NIRS

Near Infrared Spectroscopy is already well established for routine QC in feed mills. Instant analysis and reporting coupled to high through-put make NIRS attractive for screening feed for MBM adulteration/contamination. However work by the Stratfeed group showed that the limit of detection was typically 1 to 3 % MBM in a wide range of feeds (Figure7). Surprisingly the spectra of FM and MBM are quite different in spite of both being high protein meals. Detection of MBM in FM proved successful at 3, 6 and 9% MBM (Murray *et al*, 2001) largely because of the more restricted range in the FM matrix. In contrast the vast spectral range of cereal, pulses, oilseed, by-products and forage that make up plant-based feeds make for difficulties in finding robust calibration models for MBM in all types of feed matrices. Even so if NIRS is used in feed mills for screening raw material on intake it would be possible to establish outlier detection that would flag a material that was abnormal and raised suspicion. For example a set of 100