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**Investigations into blood and tissue contamination of carcasses with brain material following the use of pre-slaughter stunning methods in cattle and sheep**

# INVESTIGATIONS INTO BLOOD AND TISSUE CONTAMINATION OF CARCASSES WITH BRAIN MATERIAL FOLLOWING THE USE OF PRE-SLAUGHTER STUNNING METHODS IN CATTLE AND SHEEP


BY

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy (PhD) in the Faculty of Medical and Veterinary Sciences

February 2005


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Word count 46,736

# ABSTRACT

The emergence of bovine spongiform encephalopathy (BSE) in the UK and in other countries and establishment of its link with new variant Creutzfeldt-Jakob disease (vCJD) has initiated a review of all practices in the meat industry that may have contributed to the emergence and transmission of these diseases.
In these studies the potential contamination of cattle and sheep carcasses with brain tissues as a consequence of stunning has been investigated using a variety of applied techniques and analytic methods. These experiments demonstrated for the first time that entry of brain material into the venous circulation may occur following the use of all mechanical stunning methods currently in use or approved for use in the UK. The frequencies of brain tissue embolism observed ranged from 2 to 23 % depending upon the species and type of captive bolt gun used for stunning. Furthermore, in some cases such brain tissue emboli can be detected in the arterial blood as well as in the venous blood of stunned animals. In addition to determining the frequency of brain tissue contamination of blood, the study obtained the first experimental data on the quantity of brain tissue that enters the circulation after stunning. The extent and potential for contamination of visceral organs after stunning was investigated using detection methods based on microscopy, immunoassays and by the application of a real-time PCR assay.
The application of established anatomical and radiographic techniques demonstrated the potential for alternative vascular dissemination routes for brain tissue emboli in the carcass that have not previously been investigated in regard to stunning and slaughter of livestock.
A novel method of detecting brain tissue contamination in the venous blood using clotting time estimation in plasma was investigated in samples from cattle and sheep.

In conclusion, these studies have obtained important new information upon the potential for current stunning methods to cause contamination of sheep and cattle carcasses with brain tissues and proteins. The findings in these studies underline the importance of developing innovations and or modifications to current stunning methods to reduce the possibility of transmission of prion diseases to humans through contaminated meat.

*For Karen and Tristan*

## ACKNOWLEDGEMENTS

I wish to thank Dr. Haluk Anil for giving me the opportunity to study for a PhD degree on an interesting and topical subject. I appreciate his guidance, patience and support through-out this project including all stages of planning, experimental work and writing up.

Thanks to Dr. Chris Helps for his assistance and patience especially in the laboratory work undertaken as part of this project.

Thanks to Prof. Seth Love for the analysis of samples by microscopy and immunocytochemistry and for his thoughtful advice on the experiments undertaken.

Thanks to Dr. Francis Barr for her assistance in the radiographic studies completed.

Thanks to Justin McKinstry for his extensive assistance in experimental work and also for the excellent art work and photography that he contributed. I am also grateful for his patience and kindness in assisting me with the write-up and production of the thesis.

Thanks to Helen Weaver, Andy Phillips, Steve Gaze, Andy Shand, Marian Hiles and Tim Hillman for all their assistance in experimental work

Thanks to Sue Hughes for statistical analysis of the data.

This work was funded by the UK Food Standards Agency

## AUTHORS DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED: Ramanjan Bore

DATE: 3/6/05

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## ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| Bp | base pair |
| BSE | bovine spongiform encephalopathy |
| C | cytosine |
| CBG | captive bolt gun |
| CI | confidence intervals |
| CJD | Creutzfeldt -Jakob disease |
| Cm | centimetre |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| Ct | threshold cycle |
| CWD | chronic wasting disease |
| DEFRA | Department for Environment, Food & Rural Affairs |
| DNA | deoxyribonucleic acid |
| EC | European Commission |
| ELISA | enzyme linked immunosorbent assay |
| EU | European Union |
| FAM | 6-carboxyfluoroscein |
| FFI | fatal familial insomnia |
| FSA | Food Standards Agency |
| FSE | feline spongiform encephalopathy |
| FQ | fluorophore-quencher |
| G | guanine |
| gm | grams |
| GFAP | glial fibrillary acidic protein |
| GPI | glycophosphatidylinositol |
| GSS | Gerstmann-Sträussler-Sheinker disease |
| IM | intramuscular |
| IP | intra-peritoneal |
| IV | intravenous |
| Kg | kilogram |

| | |
|---|---|
| MBM | meat and bone meal |
| MRM | mechanically recovered meat |
| µm | micrometer |
| NF | neurofilament protein |
| OD | optical density |
| OTMS | over thirty monthes scheme |
| PCR | polymerase chain reaction |
| PNPP | p-nitrophenol phosphate |
| PrP | prion protein |
| $PrP^{c}$ | cellular prion protein |
| $PrP^{sc}$ | scrapie prion protein |
| Psi | pounds per square inch |
| rDNA | ribosomal deoxyribonucleic acid |
| RPM | revolutions per minute |
| SAF | scrapie associated fibrils |
| SD | standard deviation |
| SE | standard error |
| SEAC | spongiform encephalopathy advisory committee |
| SEM | standard error of the mean |
| SPSS | statistics package for social scientists |
| SRM | specified risk material |
| SRY | sex-determining region of the Y-chromosome |
| SSC | scientific steering committee |
| T | thymidine |
| TSE | transmissible spongiform encephalopathy |
| UK | United Kingdom |
| USA | United States of America |

# CHAPTER 1 INTRODUCTION AND REVIEW OF THE LITERATURE

## 1.0 INTRODUCTION

The epidemics of bovine spongiform encephalopathy (BSE) in cattle and of variant Creutzfeldt-Jakob disease (vCJD) in humans belong to the transmissible spongiform encephalopathy (TSE) group of diseases that were until twenty years ago relatively obscure. The acceptance that BSE is primarily a food borne disease has led to a revision of many areas within the meat industry. Although control measures introduced since the start of the BSE outbreak have largely been effective in controlling the disease, many uncertainties still exist including some that relate to routes of disease transmission to humans. The question of contamination of carcasses with potentially infected tissues at stunning and slaughter of livestock was first raised nearly ten years ago. Despite some limited work in this area, including studies involving examination of carcasses for brain tissue contamination following stunning and slaughter of livestock, the risk posed by the use of current stunning methods remains unknown. Any risk analysis is only as good as the data upon which it is based and it is important that accurate estimates are made at each critical control point of meat production. In this study the potential of various stunning methods to cause contamination of edible parts of the carcass with brain tissue is examined by the application of previously validated immunoassays and additional applied techniques.

Chapter 1

# LITERATURE REVIEW

## 1.1 TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

### 1.1.1 Historical perspective

The first description of diseases which today are classified as Transmissible Spongiform Encephalopathies (TSEs) were made in the 18th Century in England, in reference to an unusual disease that was prevalent in the sheep flock and was adversely affecting the wool industry (Schwartz, 2003). In England, this disease was given the name Scrapie to describe the frequent observation that affected sheep would rub themselves against posts and fences as if to relieve an itch. In the 19th Century, scientific investigation of this disease demonstrated neuronal vacuolation in the brains of animals that had died of Scrapie and further studies in the 1930's demonstrated the transmissibility of the disease to previously healthy sheep (Cuille & Chelle, 1936). In 1935 Scrapie was found to have been transmitted in contaminated batches of Louping ill vaccine that not only confirmed the transmissible nature of the agent but also demonstrated its unusual resistance to the formaldehyde in which the vaccine had been prepared (Gordon, 1946).

In the 1920's a rare disease, later to be known as Creutzfeldt-Jakob disease (CJD), was described in humans (Creutzfeldt, 1920; Jakob, 1921) and in the 1950's an apparently similar disease, locally called Kuru, was reported among a primitive people in what is now Papua New Guinea (Gajdusek & Zigas, 1957).

A connection between these outwardly similar diseases of humans and of Scrapie disease in sheep was suggested by a veterinary surgeon (Hadlow, 1959) and resulted in a series of experiments that demonstrated the transmissible nature of these diseases and indicated a related infectious aetiology (Gajdusek, 1977). The causative agent of these diseases remained unknown until the application of advanced biochemical techniques in the early 1980's suggested a protein coded by the hosts own genome as the infectious cause (Prusiner, 1982).

### 1.1.2 Transmissible Spongiform Encephalopathy diseases

This group of related neurodegenerative diseases are today recognised in a variety of mammalian species including humans and have been described throughout the world. Common clinical features of these diseases include a generally long incubation period, a chronic and progressive course and an inevitably fatal outcome after a variable period of illness (Taylor, 2002). More specific clinical signs are very variable and have led in the past, before the development of specific tests, to confusion in the correct diagnosis of cases. Histo-pathological examination of brain tissue from infected individuals demonstrates characteristic spongiform changes with neuronal death along with the frequent observation of insoluble amyloid-like deposits in the brain (Wells et al., 1987). Members of this group of diseases are now known to include, Bovine Spongiform Encephalopathy, Feline Spongiform Encephalopathy, Scrapie in sheep, Chronic Wasting Disease of cervids and elk, Transmissible Mink Encephalopathy of farmed mink and finally Kuru, Creutzfeldt-Jakob disease (CJD), Fatal Familial Insomnia (FFI), and Variant CJD of humans (Table 1.1).

Table 1.1 Transmissible Spongiform Encephalopathies

| Disease | Known hosts | Distribution |
|---|---|---|
| BSE | cattle | See Table 1.3 |
| Scrapie | sheep, goats | World-wide except notably Australia, New Zealand |
| Transmissible mink encephalopathy (TME) | farmed mink | North America |
| Chronic wasting disease (CWD) | mule deer, elk | North America |
| Feline spongiform encephalopathy (FSE) | cats | UK |
| Creutzfeldt-Jakob disease (CJD) | humans | World-wide |
| Kuru | humans | Papua New Guinea |
| vCJD | humans | UK, Ireland, Italy, France |

### 1.1.3 Infectious agent

Transmissible spongiform encephalopathies are thought to be caused by small, proteinaceous particles which, although apparently infectious, appear to lack nucleic acids (Prusiner, 1982). Prion theory proposes that abnormal forms of naturally occurring prion proteins are responsible for disease (Prusiner, 1998). Normal prion proteins ($PrP^c$) are naturally occurring glycosylated membrane proteins anchored in rafts through a glycosyl phosphatidyl inositol (GPI) chain (Baron et al., 2002). Nuclear magnetic resonance imaging of $PrP^c$ indicates a small globular protein of 253 amino acids in humans consisting of three ∂-helical regions, an anti-parallel ß-pleated segment and a disordered N-terminus (Collins, Lawson & Masters, 2004). $PrP^c$ is found most abundantly in brain tissue in normal individuals but also occurs in smaller quantity in other tissues including those of the reticulo-endothelial system (Foster et al., 1993[a]).

The normal function of $PrP^{c}$ remains uncertain although it seems likely that it is a copper binding protein and is important in cell survival (Vassallo & Herms, 2003). The pathogenic form of prion protein ($PrP^{sc}$) is deposited in the brain as an insoluble, relatively protease resistant iso-form of the normal prion protein. The pathogenic form of prion protein does not differ in primary amino acid sequence from normal prion protein but does have tertiary structural differences that include a higher ß-sheet content (Prusiner, 1998)

### 1.1.4 Pathogenesis of infection

The pathogenesis of TSE disease is associated with the expression and accumulation of structurally aberrant conformers of the prion protein generically called $PrP^{sc}$ (Bolton, McKinley & Prusiner, 1982). Infection is believed to occur following the formation of dimers between $PrP^{sc}$ and $PrP^{c}$ that induce a conformational change of $PrP^{c}$ into $PrP^{sc}$ (Prusiner, 1982). This conversion produces a protein that is relatively resistant to Proteinase K digestion, a feature which is frequently exploited in diagnostic studies (Meyer et al., 1999). The 'protein only' theory of prion disease has been supported by in-vitro studies demonstrating a structural change in the protein from a predominantly alpha-helical structure to one with higher beta-sheet conformation (Jackson et al., 1999). It is the progressive replacement of the normal prion protein for the abnormal $PrP^{sc}$ that is central to the pathogenesis of all prion diseases. Investigation of the role of chaperone proteins in transporting prion protein from the endoplasmic reticulum is providing new insights in to the mechanism of abnormal prion protein accumulation and deposition (Kimura et al., 2004).

### 1.1.5 Species barrier

All TSE diseases are thought to share the same pathogenesis in various species although transmission between species is known to be less efficient than that observed within a species. The basis of this so called 'species barrier' may relate to amino acid differences between prion proteins from different species (Bosque, 2002). The species barrier is an

important concept in risk analyses of BSE transmission since it may have been an important and fortuitous factor in limiting transmission of cattle BSE to humans.

## 1.2 BOVINE SPONGIFORM ENCEPHALOPATHY (BSE)

### 1.2.1 The development of disease

In the middle of the 1980's an unusual neurological disease was observed in cattle in the United Kingdom. The disease affected mainly dairy cows of 4-5 years of age. Clinical features were insidious in onset and included increased excitability and aggression and were often accompanied by abnormalities in gait (Wells et al, 1987). These signs were associated with generalised signs of disease including weight loss, reduction in milk yield and finally recumbency and death (Wilesmith et al., 1988).

The incidence of the disease increased rapidly in the following years to reach epidemic proportions in the UK (Defra, 2004) (Table 2) and subsequently to be recognised in many European countries, Japan and most recently in North America (OIE, 2004) (Table 3). Histopathological examination of brain tissue from affected animals revealed vacuolations of the grey matter neuropil and neurones in the brain stem as well as the appearance of deposits known as scrapie associated fibrils. These characteristic changes identified the disease as a spongiform encephalopathy (Wells et al., 1987).

The origin of BSE remains unknown, although it has been suggested that it originated as a sheep or bovine derived strain of abnormal prion in a single animal (Wilesmith, Ryan & Atkinson, 1991). The practice of re-cycling rendered tissues back to ruminant animals as meat and bone meal (MBM) containing the infectious agent is believed to have spread the disease to other animals (Wilesmith, Ryan & Atkinson, 1991). Much speculation has surrounded changes to the rendering process that were made in the late 1970's in the UK that may have allowed the survival of a "rogue" prion protein and allowed transmission to other animals by way of the feed (Taylor & Woodgate, 2003).

Table 1.2 Reported annual cases of bovine spongiform encephalopathy (BSE) in Great Britain (Defra, 2004)

| Year | Reported cases of BSE |
|---|---|
| 1987 | 442 |
| 1988 | 2469 |
| 1989 | 7137 |
| 1990 | 14181 |
| 1991 | 25032 |
| 1992 | 36682 |
| 1993 | 34370 |
| 1994 | 23945 |
| 1995 | 14302 |
| 1996 | 8016 |
| 1997 | 4313 |
| 1998 | 3180 |
| 1999 | 2276 |
| 2000 | 1355 |
| 2001 | 1113 |
| 2002 | 1039 |
| 2003 | 547 |
| 2004 | 201 |
| Total cases | 180,600 |

Table 1.3 Number of reported cases of bovine spongiform encephalopathy worldwide (excluding Great Britain) (OIE, 2004)

| | 1989 | 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Austria** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | - |
| **Belgium** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 3 | 9 | 46 | 38 | 15 | 7 |
| **Canada** | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| **Czech Rep.** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 4 | 1 |
| **Denmark** | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 3 | 2 | 0 |
| **Finland** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | - |
| **France** | 0 | 0 | 5 | 0 | 1 | 4 | 3 | 12 | 6 | 18 | 31 | 161 | 274 | 239 | 137 | 14 |
| **Germany** | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 2 | 0 | 0 | 7 | 125 | 106 | 54 | 14 |
| **Greece** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | - |
| **Ireland** | 15 | 14 | 17 | 18 | 16 | 19 | 16 | 73 | 80 | 83 | 91 | 149 | 246 | 333 | 183 | 47 |
| **Israel** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | - |
| **Italy** | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 48 | 38 | 29 | - |
| **Japan** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 4 | 2 |
| **Liechtenstein** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | - | - |
| **Luxembourg** | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| **Netherlands** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 20 | 24 | 19 | 4 |
| **Poland** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 5 | 5 |
| **Portugal** | 0 | 1 | 1 | 1 | 3 | 12 | 15 | 31 | 30 | 127 | 159 | 149 | 110 | 86 | 133 | 27 |
| **Slovakia** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 6 | 2 | 2 |
| **Slovenia** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| **Spain** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 82 | 127 | 167 | 44 |
| **Switzerland** | 0 | 2 | 8 | 15 | 29 | 64 | 68 | 45 | 38 | 14 | 50 | 33 | 42 | 24 | 21 | 0 |

### 1.2.2 Infectious dose

It has been estimated that up to 750,000 BSE infected cattle entered the human food chain before controls were implemented in 1988 (Anderson et al., 1996). From these infected cattle millions of people in the UK were exposed to potentially BSE infected meat. The incubation period of vCJD in humans is still unknown although estimates indicate a period of 15 to 18 years in most individuals but it is likely to be prolonged beyond this time in at least a proportion of infected individuals (Ghani, 2002). The infectious dose of BSE in humans is also unknown although studies using laboratory animal models suggest that it might be very small (Cummins et al., 2001). Infectivity of prion protein is estimated by bioassay using either transgenic mice or other laboratory species. An $LD_{50}$ of $10^{9.3}$ per gram of brain by intra-peritoneal administration has been determined by experiments using the 4PBI BSE strain isolated in C57BL/6 mice and implies that a single $LD_{50}$ infectious dose is equivalent to 0.5ng brain (Maignien et al., 1999). It is estimated that between $10^5$ and $10^9$ times as much infectivity is required for the oral route of infection as by intra-cranial injection (Prusiner et al., 1985; Kimberlin & Walker, 1989). This quantity has been compared to a minimum infectious dose of 50-µg to 500-mg of infected brain tissue (Love et al., 2000). More recent oral challenge studies with calves have demonstrated infection following ingestion of as little as 1-mg of infected bovine brain material (Konold et al., 2004).

### 1.2.3 Infectivity in tissues

The infectivity of tissues including brain and spinal cord is already well documented (Jeffrey et al., 2001), however, the potential for TSE infectivity to be present in edible parts of the carcass, including muscle, is still unclear. The infectivity of muscle tissue was not previously documented in animals or humans infected with TSE diseases (Wadsworth et al., 2001) until recently when $PrP^{sc}$ was detected in the muscle tissue of mice and hamsters experimentally infected with Scrapie (Bosque et al., 2002). In a separate study, $PrP^{sc}$ has been detected in muscle and spleen from patients diagnosed with sporadic CJD (Glatzel et al., 2003). The potential for secondary contamination with $PrP^{sc}$ of muscle tissues of beef carcasses as a result of slaughter and butchery practice is also described (Bauer et al., 1996; Anil et al., 1999; Helps et al., 2002).

Contamination of muscles with potentially BSE infected brain tissue by way of the circulation following captive bolt gun stunning of cattle has been hypothesised (Anil et al., 1999; 2001; 2002), but to date has not been demonstrated.

### 1.2.4 New strains and public health

A variety of strain types of TSE agents are known to exist and are identified by differences in the molecular mass of the $PrP^{sc}$ fragments, by the degree of glycosylation of the prion protein and upon the characteristic biological properties on transmission of the agent to laboratory animals. Indeed, it was through strain typing that convincing evidence of a link between BSE and vCJD was first made (Hill et al., 1997). The existence of new strains of BSE might have substantial implications for public health. Although recent estimates of the size of the vCJD epidemic suggest that fewer people may be affected than had originally been feared, researchers in Switzerland have noted an alarming increase in cases of sporadic CJD, raising questions of the possibility of an infectious aetiology (Glatzel et al., 2003).

As part of an active BSE surveillance program in Italy over a million cattle brain-stems were tested of which 103 were found positive by August 2003. In two of the cattle brains examined a new BSE strain was identified with very different molecular and neuropathological characteristics from typical BSE cases. Furthermore, the molecular signature of this so called bovine amyloidotic spongiform encephalopathy (BASE) showed similarities to a $PrP^{sc}$ type associated with the sporadic form of CJD observed in humans (Casalone et al., 2004).

### 1.2.5 Creutzfeldt-Jakob disease

Before the emergence of BSE in the UK, CJD and other related diseases of humans were well recorded but relatively obscure due to there rarity (Masters et al., 1979). Three variations of CJD have been recognised since its first description in the 1920's (Creutzfeldt, 1920; Jakob, 1921). These variants included a sporadic form, familial form and, more latterly, an iatrogenic form of the disease that was first highlighted by

transmission of CJD through contaminated human growth hormone treatment (Brown, 1988).

The possibility that BSE might be capable of infecting humans was largely dismissed in the early years of the BSE epidemic, although the similarity with CJD in humans was recognised soon after the epidemic in cattle was first observed (Taylor, 1989). Nevertheless, in spite of the perceived small risk to humans a National CJD surveillance unit was set up in the UK in 1990 in order to monitor CJD as a precautionary measure against any increase in CJD cases (UK CJD surveillance Unit). Unfortunately these early fears were confirmed when a novel form of CJD was observed in 1995 in the UK (Will et al., 1996). This new disease that was later named new variant CJD (vCJD), while showing many of the features of sporadic CJD differed in certain key clinical features. The age range of victims was much younger than expected (median 28 years, range 14-74), ataxia and psychiatric symptoms were more prominent and the duration of illness until death was also longer than that associated with sporadic CJD. Electroencephalogram (EEG) studies of the brains of victims were unremarkable although more recently magnetic resonance scanning (MRI) has proved helpful in diagnosis of the disease (Collie et al., 2003). All victims of this disease to date have been homozygous for methionine at codon 129 in the prion gene, suggesting an increased susceptibility to infection by this fraction of the population. Some researchers have suggested that the present distribution of the homozygous genotype may suggest that strong balancing selection at this locus occurred during the evolution of modern humans as a result of past outbreaks of TSE disease (Mead et al., 2003). A case of pre-clinical vCJD was reported from a patient who received a blood transfusion from a donor who subsequently died of vCJD. The infection was detected by a combined Western-Blot method and immunohistochemical analysis of the spleen in an individual heterozygous at codon 129 of the prion protein gene (Peden et al., 2004). This finding has confirmed earlier predictions that other genotypes may also be susceptible. On microscopic examination of the brains of victims of vCJD spongiform changes were visible in the caudate nucleus and putamen and florid plaques were identified in the cerebral cortex and cerebellar cortex (Ironside, 2003). Others brain tissues demonstrated extensive loss of neurones. All these signs were typical of the kind of pathology observed in TSE diseases of humans and animals.

At the time of writing 148 people have died of vCJD in the UK and a further 5 people have been diagnosed with the condition (UK CJD surveillance Unit). There is now compelling evidence that infection occurred by ingestion of BSE contaminated beef or from beef products, including those derived from mechanically recovered meat (Bruce et al., 1997). Recent research has raised the worrying possibility that BSE may also account for some cases of a sporadic-like form of CJD in humans (Asante et al., 2002). The finding of two cases of vCJD in people who received blood transfusions from people who subsequently contracted vCJD has given credence to reports that the agent may be transmitted between individuals through blood and blood products (Peden et al., 2004). Biopsies of tonsil and appendix have been demonstrated to offer ante mortem diagnosis of vCJD at early stages of disease before clinical signs become apparent. A recent survey of stored appendices found a single positive out of more than eight thousand tested, translating into an eventual estimate of 120 cases of vCJD per million in the UK population (Hilton et al., 2002). While great efforts are being made to prevent further infections in the population, the fear that many people may already be infected has prompted research into potential treatments. Currently several trial compounds have shown potential for slowing the formation of pathogenic prion proteins in-vitro although the results in human subjects has been less encouraging to date (Dyer, 2003).

Table 1.4 Worldwide annual deaths from new variant Creutzfeldt-Jakob disease (vCJD)

| Year | Deaths from vCJD in countries excluding the UK | Deaths from vCJD in UK |
|---|---|---|
| 1995 | | 3 |
| 1996 | 1 (France) | 10 |
| 1997 | | 10 |
| 1998 | | 18 |
| 1999 | | 15 |
| 2000 | 1 (France) | 28 |
| 2001 | 1 (France) | 20 |
| 2002 | 3 (France) | 17 |
| 2003 | | 18 |
| 2004 | 3 (France), 1 (Italy), 1 (Ireland) | 9 |

### 1.2.6 Butchery practice and variant Creutzfeldt-Jakob disease

The use of traditional butchery practices in which brains from cattle were routinely removed and used in the preparation of food products is believed to be responsible for the cluster of vCJD cases that occurred in Queniborough in the UK (Adams, 2001[a]). Brain material may also have contaminated hands and knives with infectious prions that were then inadvertently transferred to meat-cuts that entered the human food chain. These cases under-line the importance of investigating all potential routes by which brain material might contaminate meat through slaughter and butchery practice however small the perceived risk.

### 1.2.7 Transmissible spongiform encephalopathy diseases of sheep

It is known that sheep in the UK were exposed to the same BSE contaminated food as cattle during the 1980s albeit in smaller quantities (MacKensie, 2002). Experimental inoculation of several sheep genotypes with BSE infected tissues has demonstrated the susceptibility of the species to BSE (Foster et al., 1993[b]). It has been suggested that Scrapie, which demonstrates similar clinical signs to experimentally induced BSE in sheep, may be masking the presence of BSE in a small proportion of animals (Baron & Biacabe, 2001). In addition, the distribution of infective tissues in sheep experimentally infected with BSE has been shown to be much greater than that in cattle (Jeffrey et al., 2001) indicating that the entire carcass from a BSE infected sheep might be infectious. In contrast, in cattle the majority of BSE infectivity is confined to tissues of the CNS and other tissues currently designated as SRM (Defra, 2004). Despite extensive testing BSE has not been identified in the sheep population to date. Furthermore, monitoring of Scrapie incidence has shown no marked increase in recent years making it unlikely that a substantial BSE epidemic has occurred in sheep (Gravenor et al., 2000). Surveillance and testing of Scrapie cases from both recent and stored samples by sensitive tests for prion protein has revealed a small number of cases that do not fit the recognised criteria for Scrapie disease of sheep. At the same time these cases currently designated as atypical Scrapie cases appear not to be that of typical BSE of sheep (Buschman et al., 2004).

Table 1.5 Reported annual cases of Scrapie disease in Great Britain since 1993

| Year | Number of confirmed cases |
|---|---|
| 1993 | 328 |
| 1994 | 235 |
| 1995 | 254 |
| 1996 | 460 |
| 1997 | 508 |
| 1998 | 499 |
| 1999 | 598 |
| 2000 | 568 |
| 2001 | 295 |
| 2002 | 428 |
| 2003 | 439 |
| 2004 | Not available |

### 1.2.8 Bovine spongiform encephalopathy disease in other species

Several species including humans were presumed infected with BSE by the inclusion of BSE infected material in the diet before implementation of effective control measures. Pigs have been demonstrated to be susceptible to BSE infection by the parenteral route (Dawson et al., 1990) and it is likely that some British pigs were fed the same infected meat and bone rations that are thought to have spread the infectious prions to cattle. However, pigs are unusual among simple stomached animals in being resistant to BSE infection by the oral route, a finding that fortunately makes it unlikely that pigs were infected by way of BSE infected rations (Wells et al., 2003).

TSE disease has been reported in zoo animals including nyala and greater kudu and several cheetahs, lions, pumas and tigers that were presumably infected by consuming BSE infected rations (Defra, 2004).

Since 1990 approximately 89 cases of feline spongiform encephalopathy (FSE) have been identified in the UK plus additional isolated cases in three European countries. It

is thought that these cases were caused through the consumption of commercial cat food containing BSE infected tissues (Defra, 2004). A recent report describes on-going tests on the brain of a French goat that initial tests suggested had been naturally infected with BSE (Anon, 2004[b]). If confirmed, this would be the first recorded case of naturally acquired BSE in this species and must also raise further questions as to the existence of undetected BSE cases in sheep.

### 1.2.9 Transmissible spongiform encephalopathy disease in other species

Prion disease caused by agents other than that affiliated with BSE afflicts several species of animals including some that are consumed by humans. Scrapie is a neuro-degenerative disease first described in sheep (Schwartz, 2003) but also known to infect goats (Baylis & Goldman, 2004) and Moufflon (Wood et al., 1991). Scrapie is not thought to cause disease in humans although clusters of spongiform disease in countries in which sheep brains are consumed has resulted in investigations that have so far found no connection (Zilber, Kahana & Abraham, 1991). The epidemiology of Scrapie is linked to recognised polymorphisms on codons 136, 154 and 171 of the ovine prion protein gene (Hunter et al., 2003) in common with other TSE diseases in other species that also demonstrate preferential susceptibility in different genotypes.

Transmissible mink encephalopathy is a prion disease of farmed mink seen mainly in North America that is thought to be transmitted through prion infected rations (Marsh & Hadlow, 1992). In North America the epidemic of chronic wasting disease (CWD) in wild deer species appears to be increasing (Bosque, 2002). Although no evidence of transmission to humans exists, cases of prion disease among hunters who were known to have consumed wild venison have raised questions over the possibility of a link with disease in humans (Belay et al., 2001).

## 1.3 CONTROL MEASURES

### 1.3.1 Control of BSE in Animals

A range of control measures have been implemented since 1986 in the UK to prevent further infection of livestock with the BSE agent. These measures have been successful in causing a significant yearly decrease in the number of infected cattle in the UK.

#### 1.3.1.1 Notification, slaughter and incineration

In June of 1988, BSE was made a notifiable disease in the UK and in August of that year cattle suspected of having BSE were compulsorily slaughtered and destroyed by incineration with 100% compensation paid to farmers for confirmed cases of the disease. An offspring cull of all cattle confirmed to have BSE was made compulsory in 1999 (MAFF, 1999).

#### 1.3.1.2 Records, tagging and passports

Investigations and control measures instituted following the identification of BSE positive animals requires an accurate method of tracing and identifying all animals that may be infected or had contact with infected animals. Legal requirements of record keeping include maintaining a history of all animal movements between premises and records of calf births and ear tag numbers. Records must be kept for a period of at least ten years. In 1998 the cattle tracing system (CTS) was introduced to monitor the movement of all cattle born after 1996 by way of cattle passports (MAFF, 1999).

#### 1.3.1.3 Meat and bone meal ban

Rendering is the process by which waste products from the meat processing industry are converted by cooking at high temperature into tallow and meat and bone meal (MBM). The greater quantity of this MBM was fed to high producing dairy cattle as an ingredient of concentrate feed. In the late 1970's a new method of rendering was introduced which reduced the use of organic solvents in the process. It has been suggested that this change in practice may have triggered the BSE epidemic by allowing the survival of a sheep derived prion (Taylor, 1995). Epidemiological investigations of BSE indicated a point source origin for the disease of which the MBM feed was the most likely (Wilesmith et al., 1991).

A ban was placed on feeding ruminant tissues back to ruminants in 1988 in the UK and, although not immediately effective, has largely been responsible for the observed steady decline of BSE cases. Experiments have demonstrated that cow to calf transmission may account for a small proportion of new cases born after this ban was imposed (Bradbury, 1996). The continued illegal use of old feed stocks after the ban has also been cited as an additional factor for cases of BSE in animals born after imposition of the feed ban.

A further ban was placed on feeding ruminant derived Meat and Bone meal to all farm animals in 1996 along with other stringent measures aimed at eradicating BSE from the UK herd. Some European countries have been slow to implement strict control measures and in such countries the incidence of BSE continues to rise.

### 1.3.2 Control of BSE transmission to humans

Although transmission to humans was judged unlikely at the start of the BSE epidemic, some measures were taken to protect public health in the UK soon after BSE was recognised. The recognition of vCJD in 1996 (Will et al., 1996) resulted not only in the implementation of much more stringent measures to control BSE but also in an EU ban on UK beef exports, with devastating economic effects upon the UK beef industry.

### 1.3.2.1 Inactivation of TSE agents

The agents of TSE disease are extremely resistant to physical and chemical procedures that inactivate other disease agents. Standard decontamination procedures such as exposure to ultraviolet and ionising radiation are not effective in deactivating the infectious form of prion protein. The use of chemical disinfection methods have included, 1M sodium hydroxide, sodium hypochlorite at concentrations of 2000 ppm and autoclaving in water at 134°C that reduced infectivity by > 5.6 $\log_{10}$ lethal doses (Fichet et al., 2004). Finding effective and economic deactivating procedures is of considerable practical importance for cleaning of abattoir equipment such as the bolt of captive bolt guns, saw and knife blades and contaminated surfaces. Contamination of surgical instruments with potentially prion-infected tissues has created difficult dilemmas for surgeons. The safest option is thought to be the single use of all surgical instruments, however the cost of this option would be prohibitive. A trial introduction of single use plastic instruments was shown to have far more mortality associated with their use than the risk associated with contracting vCJD through surgery (Frosh, Joyce & Johnson, 2001).

### 1.3.2.2 Specified risk materials (SRM)

As a further measure to prevent exposure of humans to the BSE infectious agent, tissues believed to contain up to 99% of infectivity were banned from human use in the UK from 1989. These tissues originally designated Specified Bovine Offal (SBO), included brain, spinal cord, tonsil, thymus, spleen and intestine from all cattle over 6 months of age. In the following years this ban was broadened to include other bovine tissues as well as inclusion of sheep and goat heads, spleens and spinal cords and is now designated Specified Risk Material (SRM). In practice it is not possible to easily remove the spinal cord or brain, so in effect it is the head and spine that are removed from carcasses and incinerated. In a recent amendment to SRM regulations (EC regulation 149/2004) small bone chips from the spinous and transverse processes from specified areas of the bovine vertebral column removed with the meat during boning operations will no longer be classified as SRM (Anon, 2004[a]). All tissues designated as

SRM are removed from carcasses at slaughter and dyed and stored separately prior to destruction by incineration.

Table 1.6 Tissues currently designated as specified risk material (SRM) in the UK (EC No 999/2001) (FSA, 2004)

| *Species* | *Age* | *SRM* |
|---|---|---|
| Bovine | >6 months | Skull, brain, eyes (head excluding the tongue), tonsils, spleen, spinal cord |
| Bovine | Calves of any age | Intestine (from duodenum to rectum), thymus, tonsils, mesentery, spleen |
| Ovine/caprine | Permanent incisor tooth | Skull, tonsils, brain , spinal cord, eyes |
| Ovine/caprine | All | Spleen, ileum |

1.3.2.3 Over thirty months scheme (OTMS) rule

Nearly all clinical cases of BSE have been in animals over thirty months of age and no reported cases below this age limit have occurred in the UK since 1996 (Bradley, 2003). The long incubation period of BSE in cattle (mean 60 months) and experimental evidence that infectivity is restricted to the distal ileum in animals that are under 32 months of age has re-enforced the belief that only a negligible risk exists to the consumer from animals less than 30 months old. All cattle older than thirty months are slaughtered at designated abattoirs and incinerated. Exceptions to this rule include some grass fed cattle in the Beef assurance scheme that are authorised for human consumption up to 42 months of age. In 2001 there were 8 OTMS designated abattoirs out of a total of 394 in Britain. At this time, fears were expressed over the possibility of transmission occurring at the abattoir since cattle for human consumption were also slaughtered at some of these facilities although on different days (Adams, 2001[b]). The resistance of the prion protein to normal disinfection procedures would seem to make these concerns

justified in light of the known potential for wide dissemination of neural tissues within the abattoir environment (Helps et al., 2004; Prendergast et al., 2004).

The decline of BSE cases in the UK combined with the development of sensitive post-mortem tests has initiated a revision of the OTMS rule. It has been proposed that all cattle slaughtered over 30 months of age will be tested for BSE by an accredited assay before entry into the food chain in line with current EU legislation (EC No. 999/2001). It is estimated that the replacement of the OTMS could save up to £300m a year in the UK. To date the scheme has cost around £3bn. a year to implement since its inception (FSA, 2004).

### 1.3.2.4 Changes in stunning and slaughter practice

Based on evidence that demonstrated a risk of contamination of the carcass with brain tissue after stunning (Garland, Bauer & Bailey, 1996; Anil et al., 1999) the practice of pithing after stunning was banned in 2001 and air-injection pneumatically-activated captive bolt guns are no longer recommended for use in the UK. Pithing involves the introduction of a flexible rod into the bolt-hole created by the penetrating CBG and then directed through the foramen magnum into the spinal canal to maximise destruction of the brain and spinal cord. The practice has benefits both for animal welfare and for worker safety but may result in an increased risk of carcass contamination with brain tissues (Anil et al., 1999). In addition the Scientific Steering Committee (EC, 2002) suggested that more investigation should be conducted into the prevalence of neural embolism after stunning with captive bolt gun devices currently used in the UK.

### 1.3.2.5 Ban on the use of mechanically recovered meat (MRM)

Mechanically recovered meat refers to meat removed from the skeleton, including the spinal column, after the application of conventional carcass de-boning techniques. This practice is associated with a potential risk of removing SRM tissues including the dorsal root ganglia that are known to harbour infectivity in a BSE infected carcass (Jeffrey et al., 2001).

MRM was traditionally used in the production of burgers, sausages and other meat products prior to the introduction of a ban in the UK in 1995. This ban was extended within the EU in 2001 to include MRM from bovine, ovine and caprine skeletons. Epidemiological investigations of MRM suggest that the production of beef MRM in the UK peaked at 5000 tonnes in 1987 and was around 2000 tonnes at the time of the ban in 1995 (Cooper & Bird, 2002). A study of the effectiveness of the SBO ban on reducing BSE transmission by MRM found that the SBO legislation in 1989 contributed only a 6% reduction in infectivity from beef MRM at that time. Remaining infectivity in MRM, even after the SBO ban, arose from inclusion of small quantities of spinal cord material and from the dorsal root ganglia that may have been removed with the meat (Cooper & Bird, 2002). A recent study has investigated the use of ultra-high pressure to inactivate experimental prion infectivity in processed meats with significant reductions in BSE infectivity in samples as compared controls (Brown et al., 2003). The development of methods of prion inactivation applicable to the food industry could increase the safety of processed meat products.

### 1.3.2.6 Active surveillance and post-mortem testing for BSE

In the EU, all cattle over thirty months of age that are intended for human consumption must be tested by an authorised rapid post-mortem test. Any carcass that is found positive is incinerated along with the carcass in the line before and the two in the line after (Bradley, 2003). The basis for this decision stems from research indicating that 99.95% of cattle demonstrating clinical sings of BSE are over thirty months of age. Below thirty-two months of age only the distal ileum contains detectable infectivity (Bradley, 2003). In the UK all OTMS cattle are currently disposed of according to UK OTMS regulations, however it is expected that the OTMS rule will be phased out in 2005 in favour of post-mortem testing. Post-mortem tests are based on sensitive immunoassays (Bird, 2003). Tests that are currently authorised by the EU commission include:

- The Prionics blotting system
- Enfer ELISA (Abbott)
- CEA ELISA systems (BioRad, Marnes-la-Coquette, France)

All three of these tests are believed to approach 100% in terms of sensitivity and specificity (Dealler, 2004).

#### 1.3.2.7 Ante-mortem testing for BSE

Due to the current practice of testing only a proportion of animals entering the food chain it is possible that sub-clinically infected animals under thirty months of age may enter the food chain undetected. Although such animals are likely to be less infective at earlier stages of the disease these animals might still pose a risk for the consumer. Extensive research has been invested in the development of effective ante-mortem tests that can detect BSE before characteristic clinical signs appear. Serum from BSE infected cattle and from healthy controls was analysed by a combination of infrared spectroscopy and multivariate pattern recognition analysis with a reported sensitivity and specificity for BSE diagnosis of 96 and 92% (Lasch et al., 2003). In a separate study, antibodies to Acinebacter bacteria and bovine brain peptides have been compared in BSE infected and in healthy control animals in a study to develop an ante-mortem test (Wilson et al., 2003). Other researchers have attempted to record a perturbation of heart rate among sub-clinically BSE infected cattle since pathological changes in the brain stem of infected animals may modulate heart rate variability (Promfrett et al., 2004). A recent report describes gait changes in BSE infected cattle that may be used as a useful indicator for BSE suspects (Braun et al., 2004). Despite promising results from these and other studies a reliable ante-mortem test for BSE has not yet been developed.

## 1.4 PRE-SLAUGHTER STUNNING

### 1.4.1 Stunning of cattle and sheep in the UK

Stunning animals before slaughter aims to induce a state of unconsciousness so that slaughter may be carried out without pain and suffering for the animal. In the UK, legislation protects the welfare of animals to be slaughtered by requiring the use of an approved method of stunning, exceptions include those animals slaughtered by religious methods for such as Muslim (Halal) and Jewish (Shechita) markets (Defra, 1995).

Stunning methods of sheep and cattle in the UK include electrical stunning and the use of mechanically operated devices.

### 1.4.2 Stunning methods

#### 1.4.2.1 Electrical stunning

Electrical stunning is used by approximately 70% of sheep slaughter plants but is not widely utilised for cattle stunning in the UK. Currently six plants in the UK use this system as a stunning method for cattle and its popularity may increase in future. Stunning of cattle is performed by the application of three electrical cycles comprising a head-only stun followed by a cardiac cycle and finally a spinal discharge cycle (Wotton et al., 2000) except for Halal slaughter for which head only stunning is applied. While electrical stunning is used routinely and effectively in New Zealand, where the system for cattle was developed, cost and welfare problems have so far limited its widespread implementation in the UK although it is commonly used in other European countries including Germany.

#### 1.4.2.2 Gas stunning and slaughter

The use of carbon dioxide and inert gases is used effectively in the stunning and slaughter of pigs and chickens (Gregory, Moss & Leeson, 1987; Raj, 1998). However, the method has not proven adaptable for use in cattle because of the large size of the animals and in sheep because of the potential for gas absorbency of the fleece.

#### 1.4.2.3 Captive bolt stunning

When used correctly, captive bolt guns induce an instantaneous stunned state by the application of percussive forces to the skull. Stunning by captive bolt involves the transfer of the kinetic energy of the bolt to the animal's head, which causes severe disruption of normal electrical brain activity and at the same time produces extensive tissue damage and haemorrhage (Blackmore, 1979). If operated optimally the use of

CBGs for sheep and cattle provide a cheap, efficient and welfare friendly method of stunning prior to slaughter. The emergence of BSE and vCJD and the possibility that CBGs may sometimes cause contamination of the carcass with infectious prions have called into question the safety of mechanical stunning methods for livestock (Anil et al., 1999; 2002). In the UK, captive bolt gun equipment is no longer subject to firearms legislation and consequently is readily available to those involved in the routine culling of livestock (DEFRA, 1995). The banning of captive bolt gun use would have serious welfare implications for the slaughter of livestock since no similarly efficient and available method of stunning currently exists.

#### 1.4.2.3.1 *Captive Bolt Guns used for stunning of cattle and sheep*

Captive bolt guns employed for stunning cattle comprise both penetrating and non-penetrating devices. Both types of device comprise a steel bolt retained within the barrel of the gun by a flange and is buffered by special sleeves which absorb the impact on firing and ensure that the bolt returns to its original position. When the gun is fired the expansion of gases produced by the detonation of a cartridge drives the bolt out of the barrel at speeds of 40 to 50 m/s for a distance of approximately 80-mm (HSA, 1998). Penetrating guns are designed to drive the bolt of the gun through the skull and into the brain (Figure 1.1). Non-penetrating devices are equipped with a mushroom shaped bolt end, which impacts the animal's head (Figure 1.2). These non-penetrating CBG devices are not designed to enter the animal's brain but nevertheless have been shown to be as effective as penetrating devices in inducing a stunned state (Daly & Whittington, 1989). Sheep are stunned using only captive bolt guns of the penetrating type in the UK. Both cartridge-activated and pneumatically-activated guns are used.

Figure 1.1 Schematic diagram of a non-penetrating captive bolt gun used for cattle stunning

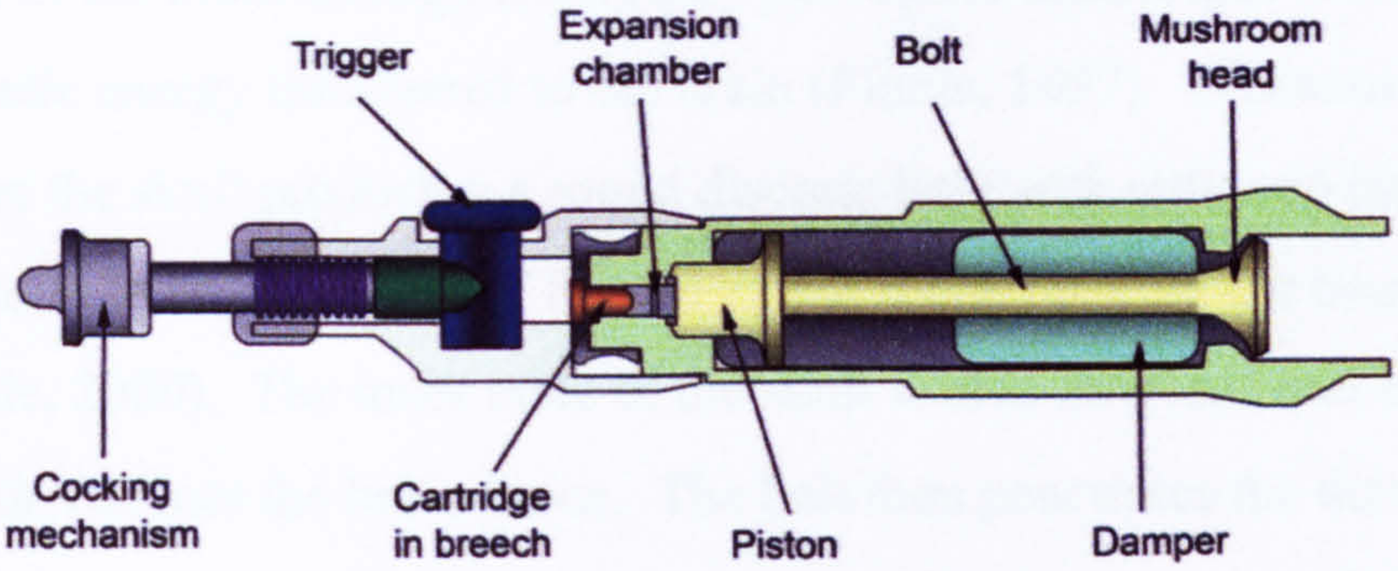
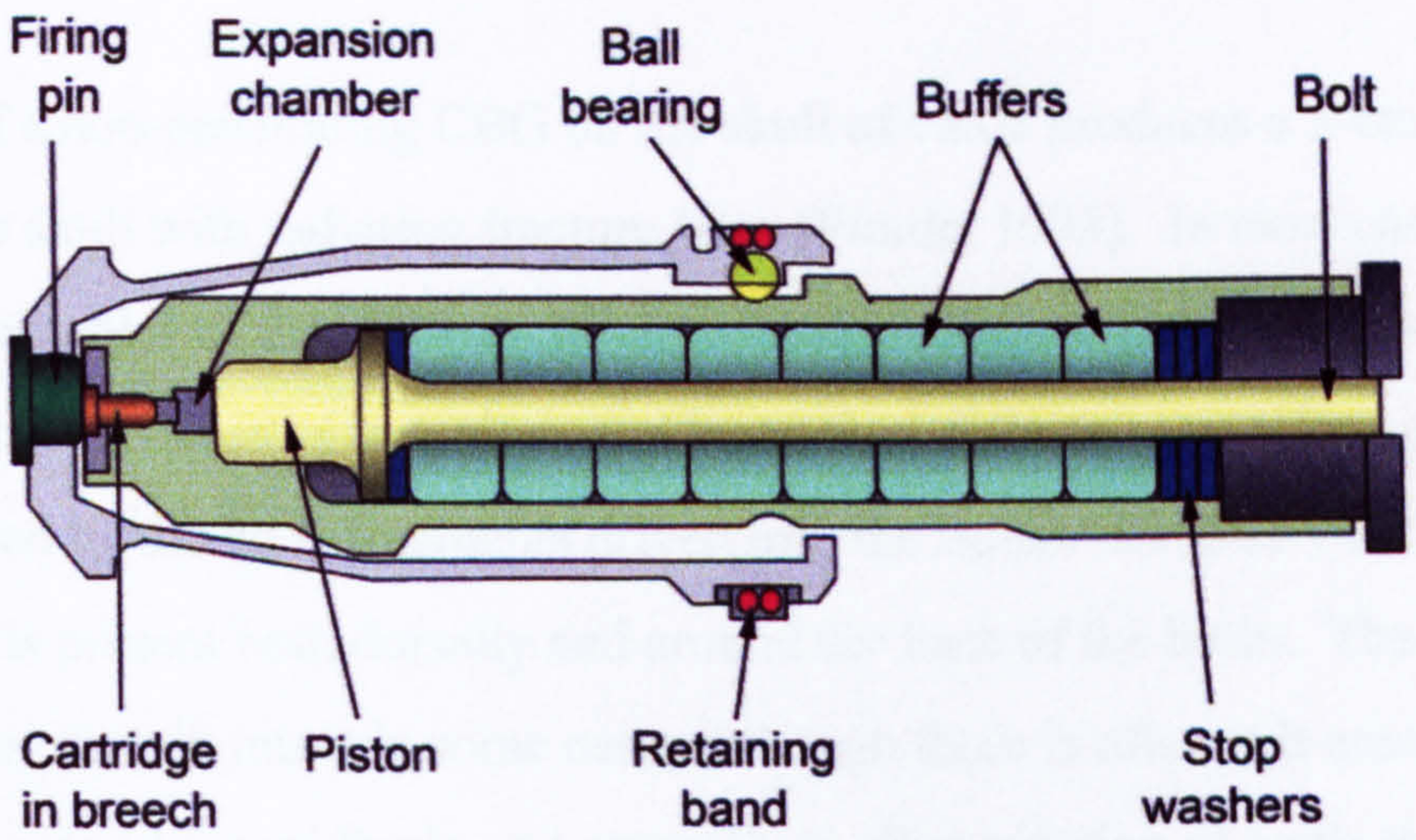



Figure 1.2 Schematic diagram of a penetrating captive bolt gun used for cattle stunning



#### 1.4.2.3.2 *Trauma induced by penetrating and non-penetrating captive bolt devices*

The magnitude of the brain damage induced by percussive stunning is determined by the quantity of kinetic energy transferred to the brain (Finnie, 1997). Penetrating CBGs initially fracture the skull producing a round discrete hole with either no attendant fractures or with fine fracture lines of limited extent radiating from the bony deficit in the skull (Finnie, 2000). The inner table of the skull is also fractured and bone fragments are driven into the brain tissue. The bolt then penetrates the dural membranes surrounding the brain and creates a permanent haemorrhagic wound track in the brain tissue. Considerable laceration and crushing of tissue is caused by the passage of the bolt in addition to rupture of the vessels of the venous sinuses. Severe damage also occurs in areas remote from the passage of the bolt including severe damage to the cerebellum and brainstem and considerable sub-arachnoid and intraventricular haemorrhage (Finnie, 1993; Farag, 2002)

The impact of a non-penetrating CBG on the skull of cattle produces a 3-cm depressed fracture of the skull with radiating fracture lines (Finnie, 1995). In most cases both the inner and outer tables of the skull are fractured and multiple fragments of skull are driven deep into the brain. Contusions and laceration of tissue are evident much of which is caused by the bone fragments driven into the tissue. Extensive sub-arachnoid haemorrhage is present both dorsally and around the base of the brain. The dural membrane may remain intact in some cases although there is often sub-arachnoid haemorrhage around the midbrain and cerebellum. Examination of cattle brains after stunning with both types of CBG suggests that comparable trauma to tissues and blood vessels are produced (Farag, 2002). The results of this recent study are in contrast to the findings of an earlier study, which found sub-arachnoid haemorrhage but no brain contusions after use of a non-penetrating CBG on cattle (Finnie, 1995).

Figure 1.3 A bovine skull and brain following non-penetrating captive bolt gun stunning demonstrating tissue trauma and haemorrhage

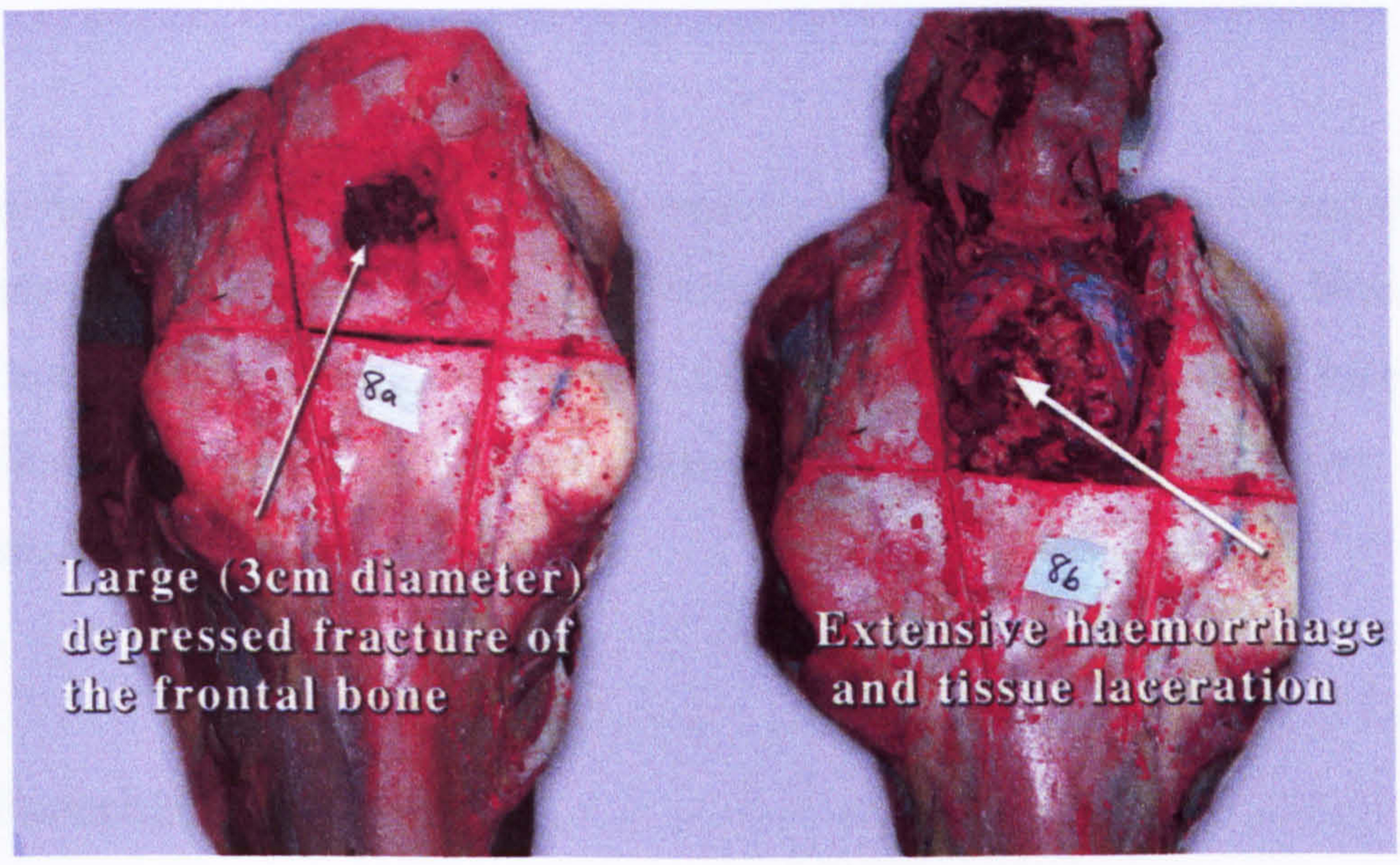
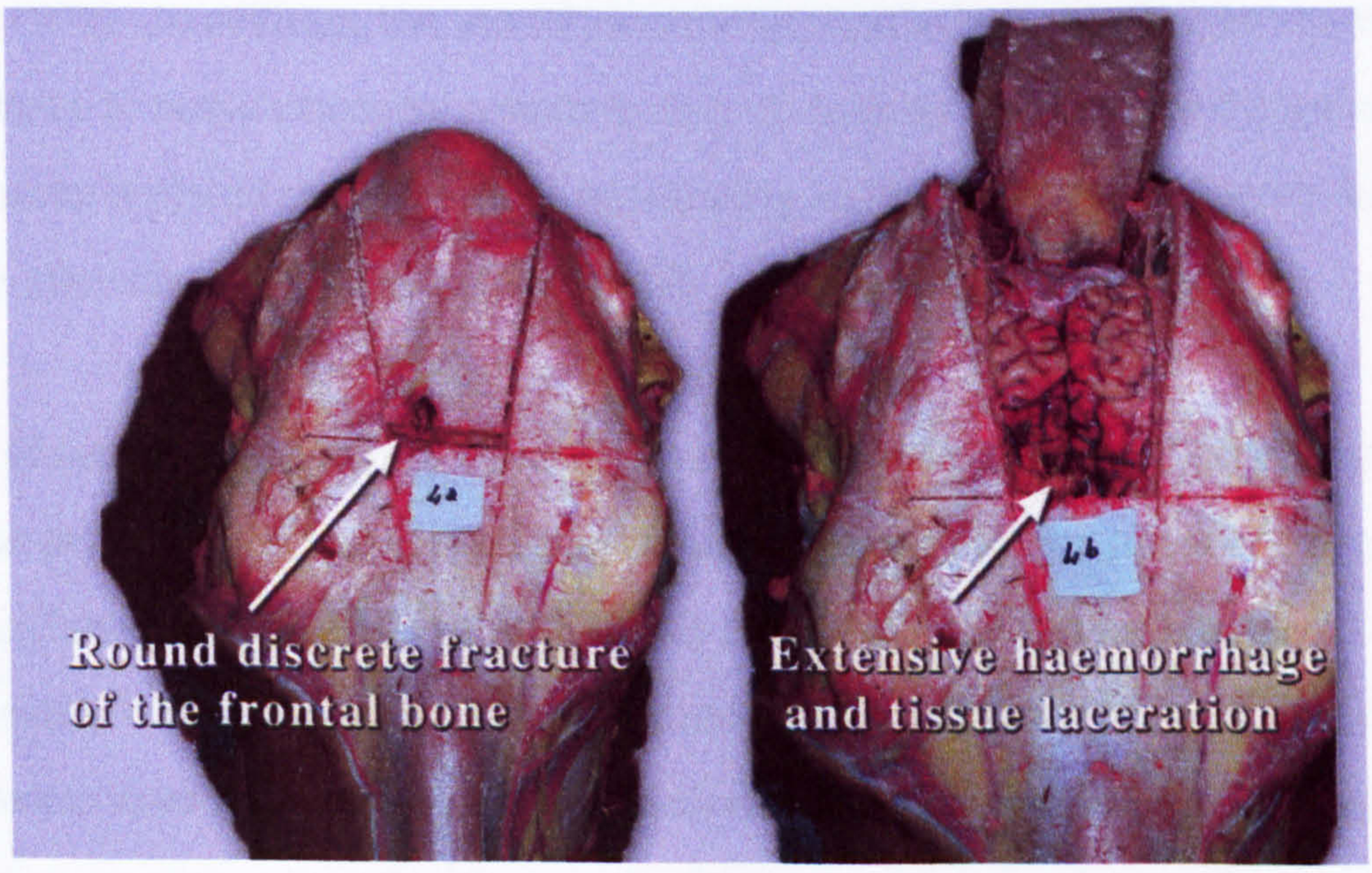



Figure 1.4 A bovine skull and brain following penetrating captive bolt gun stunning demonstrating tissue trauma and haemorrhage



## 1.5 CNS TISSUE CONTAMINATION AT STUNNING AND SLAUGHTER

### 1.5.1 Potential for carcass contamination at stunning and slaughter in sheep and cattle

The stunning and slaughter of livestock provides the opportunity for contamination of the carcass with a variety of potentially infectious agents (Anil et al., 1999; Buncic et al., 2002). Infectious agents including bacteria present on hides and hooves, in body fluids or in the tissues themselves may contaminate the carcass by the use of invasive procedures at stunning and slaughter.

#### 1.5.1.1 Bacterial contamination

All operations at stunning and slaughter of livestock in which the skin and tissues are penetrated have the potential to introduce bacteria onto edible parts of the carcass. The practice of sticking, in which a knife is used to severe major blood vessels in the neck or chest, has previously been shown to allow bacteria to enter the circulation (Mackey & Derrick, 1979). In addition, the use of penetrating captive bolt stunning has been demonstrated to cause extensive contamination of muscles and peripheral organs with a marker bacteria placed on the bolt of a penetrating captive bolt gun or directly into the bolt-hole after stunning (Buncic et al., 2002)

The use of penetrating CBGs is known to cause spread of pathogenic bacteria from the hide, coat and tissues into the blood circulation. Furthermore, the use of a marker bacterium introduced by way of the CBG has demonstrated not only extensive contamination of the carcass but also of the abattoir environment (Daly et al., 2002). Some studies have attempted to draw inferences upon the potential of brain tissue fragments to be disseminated in the carcass from experiments using marker bacteria to model the movement of the brain tissue in the blood (Prendergast et al., 2004). However the innate differences between the morphology of bacteria and that of brain tissue fragments dislodged by stunning must raise questions as to the validity of this approach.

### 1.5.1.2 CNS tissue contamination

Carcass splitting has been identified as an important contributor of carcass contamination with CNS tissue (Helps et al., 2004). Further contamination may occur by carriage of brain tissue fragments in the circulation as a result of mechanical stunning methods

#### 1.5.1.2.1 *Carcass splitting*

All areas of abattoir practice have been examined to identify areas in which such contamination might occur. Carcass splitting has been demonstrated to cause contamination of the cut surfaces of the carcass with CNS material (Helps et al., 2002) prompting research into other methods of carcass dressing to avoid damaging the spinal cord. Proposals have included the use of a hot boning method already used in some countries in which the meat is removed from the carcass without prior chilling and without the need for carcass splitting. An oval saw has recently been developed that allows removal of the intact spinal cord and surrounding vertebrae. This saw design is currently undergoing trials to evaluate commercial viability (Mr. Andy Knight, Silsoe Institute, UK, personal communication).

#### 1.5.1.2.2 *Captive bolt gun stunning*

The use of air-injection pneumatically-activated CBGs was stopped in the UK after research suggested an increased risk of carcass contamination by brain tissue fragments (Anil et al., 1999). It is thought that such tissue fragments are forced into the venous circulation by the high intra-cranial pressures created by injecting air into the cranium (Garland, Bauer & Bailey, 1996; Anil et al., 1999). Before this risk became apparent air-injection pneumatically-operated CBGs were used in the UK from 1980 and in the USA since 1982 (Roth, 2001). The contamination of edible parts of the carcass following the use of CBGs without air-injection facility has not been documented in cattle despite a previous trial on small numbers of cattle (Anil et al., 1999). It has been surmised that if brain tissue fragments were to enter the circulation such fragments would be voided in the blood at exsanguination or would become trapped in the heart or

lungs (Bradley, 2003). In either event, contamination of other parts of the carcass with brain tissue would be unlikely. However, this hypothesis has not been demonstrated by experiment or proven by observation. Although it is true that the majority of reports of brain tissue emboli describe fragments of brain tissue in the heart or vessels of the lungs the potential of small microscopic fragments to penetrate the lungs is unknown.

#### 1.5.1.2.2.1 *Environmental CNS tissue contamination*

CNS tissue may also contaminate the abattoir environment, equipment as well as the workers themselves. In a study in which a selected bacterial species was used as a marker for CNS tissue, the marker bacterium was detected on the captive bolt, around the bolt hole on the animals head and also on the hands and aprons of operatives after stunning (Prendergast et al., 2004). While such contamination may appear unavoidable, the risk involved if a BSE infected animal were to be processed might be unacceptable. Head contamination with brain tissue leaking from the bolt-hole may be reduced by the use of a rubber bung in the bolt-hole after stunning (Daly et al., 2002). In a recent study an average 0.6-g of brain tissue was recovered from the area around the bolt-hole after penetrating CBG stunning of cattle (Magill, 2004).

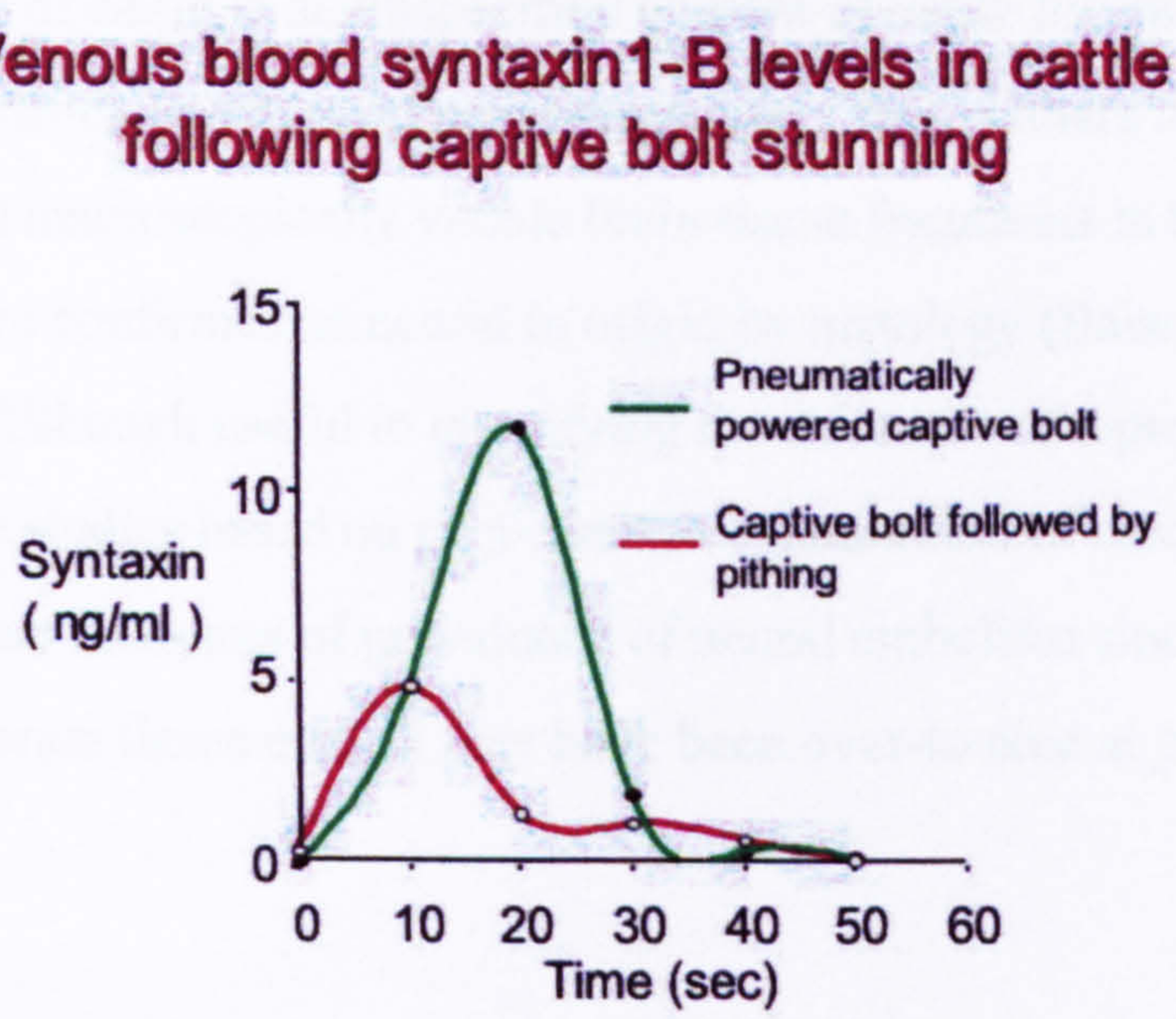
## 1.6 HEAD TRAUMA AND ENTRY OF BRAIN TISSUE FRAGMENTS AND PROTEINS INTO THE VENOUS CIRCULATION

### 1.6.1 Leakage of brain proteins into the circulation after head trauma

Measurement of serum levels of myelin basic protein, a CNS specific protein, has previously been investigated as an aid for assessment of patients after head trauma. Results suggested that following severe head trauma the blood brain barrier is breached allowing entry of some brain proteins into the circulation (Thomas, Palfreyman & Ratciffe, 1978). A variety of CNS proteins have been investigated in similar studies including S-100β (Raabe et al., 1998). Recent studies have demonstrated that even relatively mild head trauma, such as sustained by soccer players, can cause elevation of

S-100β protein in the venous blood (Mussak et al., 2003). In a recent study of CBG stunning in cattle elevated levels of the pre-synaptic protein Syntaxin 1B were detected in some samples within the first thirty seconds of stunning. In this same study good correlation was obtained between the Syntaxin assay and the results of analysis of the same samples by microscopy and immunohistochemistry in which fragments of brain tissue were actually visualised (Anil et al., 2002). The results of these studies would suggest that elevation of some brain protein markers in the blood may be associated with fragments of brain tissue dislodged by severe head trauma.

Figure 1.5 Graph demonstrating the variation in concentration of a brain specific protein in jugular blood samples taken following the application of two stunning treatments (Anil et al., 2002)




### 1.6.2 Brain tissue emboli in the circulation following severe head trauma

Pulmonary embolism following head trauma consisting of fat, marrow, skin, foreign bodies and brain are an uncommon finding on post-mortem examination (Torry, 1987). Brain tissue pulmonary embolism is an even rarer event, although cases are described in the literature following traumatic forceps delivery of neonates (Tan & Hwang, 1976) and from a variety of other severe head injuries. An early study of 213 cases of severe

head injury in humans gave an incidence of brain tissue embolism of 2% (McMillan, 1956). A more recent retrospective and prospective study of cerebral tissue pulmonary embolism after head trauma estimated an incidence of 10% (Collins & Davis, 1994). A case study of a single victim of head trauma who died some 32 hours after falling from a ladder found significant pulmonary embolism of brain tissue that showed significant tissue reaction and haemorrhage upon histopathologic examination (Torry, 1987). This unusual finding of vital brain tissue indicated that the embolism occurred some time after the initial trauma and has interesting implications for the timing and mechanism by which entry of brain tissue into the circulation might occur following severe head injuries.

### 1.6.3 Reported prevalence of brain tissue embolism after captive bolt stunning

Garland, Bauer and Bailey (1996) described an estimated incidence of 2.5-5% of neural emboli in the lungs of cattle in an unspecified number of cattle following the use of an air-injection pneumatically-activated penetrating type CBG. Others have shown that 7 of 220 animals had macroscopically visible brain tissue fragments in the pulmonary arteries, which were confirmed as neural in origin by histology (Bauer, Garland & Edwards, 1996). Although useful in identifying the existence of a problem with captive bolt stunning these studies based on post-mortem examination of carcasses are likely to have been inaccurate measures of prevalence of neural embolism since many carcasses with microscopic brain tissue emboli may have been over-looked at post-mortem examination.

These initial findings were made in the USA and workers there were quick to suggest that their findings might indicate a risk factor for contamination of meat at stunning with $PrP^{sc}$ in the UK (Garland, Bauer & Bailey, 1996). This suggestion was at first denied (Taylor, 1996) and initial studies in the UK conducted on ten cattle stunned with a conventional captive bolt gun demonstrated no embolism of cerebral tissue, a subsequent study of 210 cattle also found no evidence of neural embolism (Munro, 1997). In a related study, the hearts of several thousand cattle were examined for evidence of neural embolism after stunning and slaughter, with only 2 of 1050 confirmed as containing brain tissue emboli after the use of the air-injection pneumatically-activated CBG (Schmidt et al., 1999[b]).

In a novel approach, workers at Bristol University examined jugular venous blood collected immediately following stunning in cattle using a variety of stunning devices. The results of this study indicated that 4 of 15 cattle stunned with an air-injection pneumatically-activated device showed neural emboli in the blood collected, a single animal stunned by conventional captive bolt followed by pithing was also found to have brain tissue in jugular samples (Anil et al., 1999). The identification of the tissue detected in these later studies was confirmed by two analytic methods. The first involved the use of a capture ELISA for a brain specific protein and the second employed validated microscopic and immunocytochemical methods for staining and visualising the neural emboli with antigen-specific antibody stains (Love et al., 2000).

In a similar experiment to investigate neural embolism after stunning in sheep, brain tissue was detected in two of fifteen sheep stunned with cartridge or pneumatically-activated penetrating CBGs respectively. No evidence of brain tissue emboli was detected in the small volumes of aortic blood examined in this experiment (Anil et al., 2001). Based on the available data, the Scientific Steering Committee (SSC) of the EC ranked various stunning methods. The order of the perceived risk of causing brain tissue to enter the circulation after use of mechanical stunning methods (EC, 2002) is given below:

- Pneumatic stunner that injects air
- Pneumatic stunner that does not inject air
- Captive bolt stunner with pithing
- Captive bolt stunner without pithing
- Non-penetrating stunner or electronarcosis (Absent or negligible risk)

It was also suggested that further research was needed to investigate stunning and brain tissue contamination of the carcass.

### 1.6.4 Mechanism of entry of tissue into the circulation

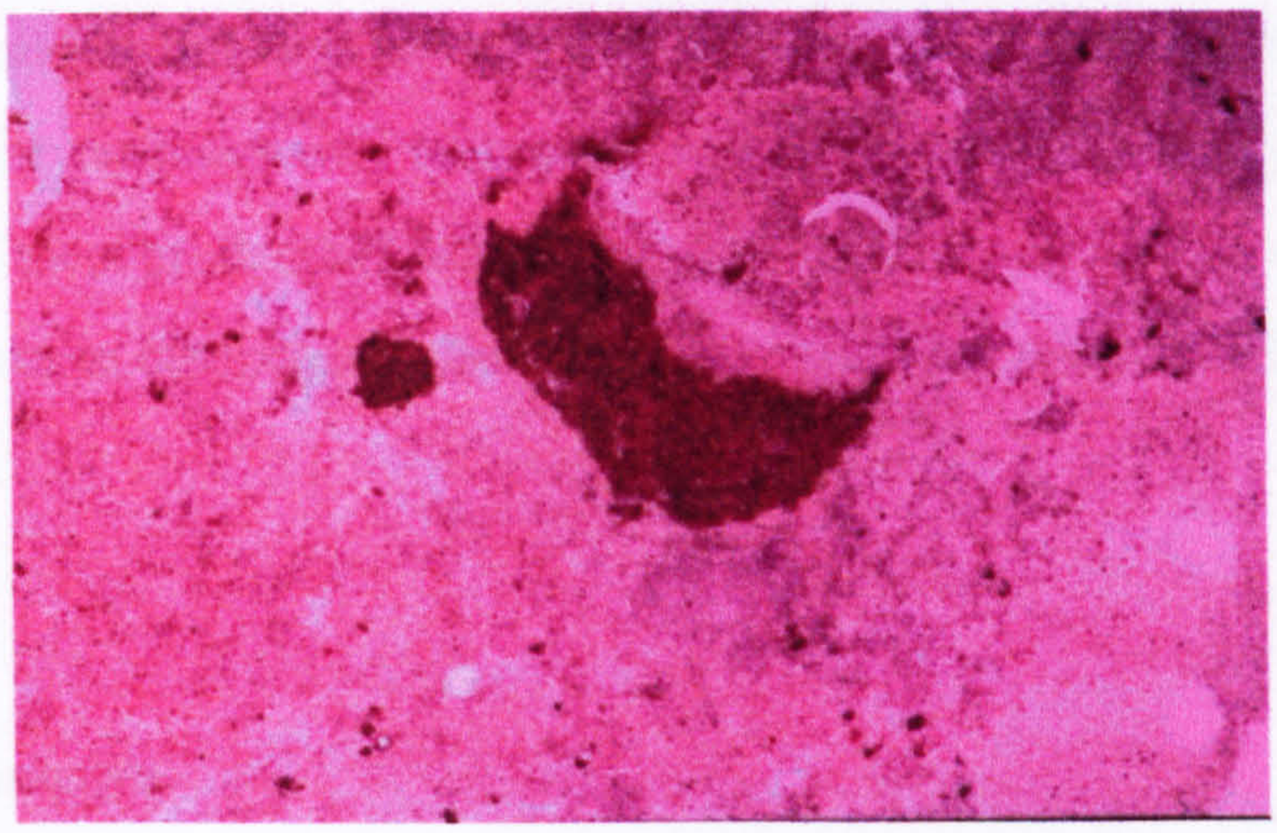
Collins and Davis (1994), in post-mortem studies found that the dura mata was intact in 70% of cases of cerebral tissue embolism suggesting that rupture of the dura and or large venous sinuses is not always necessary for entry of brain tissue fragments into the circulation. In a separate study post-mortem examination of two head trauma victims demonstrated a large cerebral venous defect in each case as the probable site of entry of brain tissue into the systemic circulation (Ogilvy et al., 1988). Although the mechanism of entry of brain tissue into the circulation has not been investigated in reference to CBG stunning of livestock, observations have been made of extensive rupture of dural membranes and cerebral venous sinuses following the application of both penetrating and non-penetrating CBG stunning of cattle (Finnie, 1993; 1995, Farag, 2002). In addition, extensive laceration of brain tissue suggests that brain tissue trauma and blood vessel rupture are contributing factors for embolism of brain tissue.

### 1.6.5 Size and quantity of brain tissue fragments that may enter the circulation at stunning

The quantity of brain material that may enter the venous return at stunning is a critical factor in any risk analysis of captive bolt gun stunning of livestock and contamination of the carcass with potentially infected tissues. Brain tissue fragments ranging from a few millimetres up to a maximum of 14-cm have been found in the pulmonary arteries of cattle after use of an air-injection pneumatically-activated CBG (Garland, Bauer & Bailey, 1996). In a separate report, segments of spinal cord as large as 10 to 13-cm were observed in the right ventricle of a single carcass after use of a similar type of CBG (Schmidt et al., 1999[b]). Fragments of brain tissue as large as 3-cm have been observed in the liver, kidney and right ventricle of the heart of a single cow after the use of an air-injection pneumatically-activated CBG in a post-mortem study of carcasses (Roth L., 2001). An examination of jugular blood samples taken from four cattle after the use of an air-injection pneumatic CBG and in a single cow after use a penetrating cartridge-activated CBG followed by pithing revealed fragments of brain tissue up to 50-µm in diameter (Anil et al., 1999). In a related study fragments as small as 5-µm were observed in jugular blood samples from sheep after use of penetrating CBGs (Anil

et al., 2001). All descriptions of brain tissue emboli in blood or in organs to date have relied on either sampling of small quantities of blood (Anil et al., 1999; 2001) or upon post-mortem observations (Garland, Bauer, Bailey, 1996; Schmidt et al., 1999[b]). Neither method has been able to quantify the total amount of brain tissue that can enter the circulation after stunning.

Figure 1.6 Fragments of brain tissue in bovine venous blood detected by microscopy and stained by immunocytochemical methods (Anil et al., 1999)



## 1.7 CNS TISSUE DETECTION METHODS

### 1.7.1 Macroscopic examination

Brain tissue fragments have previously been identified in the pulmonary arteries at post-mortem following severe head injuries (Ogilvy et al., 1988). Macroscopically visible pieces of brain tissue have been observed the pulmonary arteries of cattle after stunning (Bauer, Garland & Edwards, 1996) and in a related study segments of spinal cord up to 13-cm in length were identified in the hearts of 2 cattle on post-mortem (Schmidt et al., 1999[b]).

### 1.7.2 Histopathology/microscopy

Histopathological examination of emboli found in the pulmonary arteries of human subjects has been used to identify tissue suspected to be neural in origin (Collins & Davis, 1994). The use of routine stains including haemotoxylin and eosin may assist identification however identification of tissues by histopathology alone maybe unreliable (Davis et al., 1991).

### 1.7.3 Cholesterol measurement

Cholesterol is found in high concentration in tissues of the CNS and analysis of cholesterol content has previously been suggested as a means of identifying CNS contamination of meat products (Lücker et al., 1999). The presence of cholesterol in high concentrations in other tissues including liver and yolk makes confirmation by this method difficult.

### 1.7.4 Immunoassays

More accurate and sensitive techniques of CNS tissue confirmation have involved the development of specific antibodies raised against CNS specific proteins. A solid phase radioimmunoassay has reliably identified antibodies raised against a panel of proteins including myelin, myelin basic protein and glial fibrillary acidic protein (GFAP) (Linthicum et al., 1981). The use of ELISA based on CNS specific antigens is well documented to be reliable means of CNS tissue identification (Schmidt et al., 2002; Love et al., 2000). Numerous brain tissue antigens have been used to stain sections suspected to be CNS tissue. In recent studies, Syntaxin 1B (Anil et al., 1999; Love et al., 2000) and GFAP (Helps et al., 2002) have been used to positively identify CNS tissue contamination of carcasses.

### 1.7.4.1 Syntaxin 1β

Syntaxin 1β, a 35KD strongly membrane bound protein of CNS cells has been used as the antigen in an ELISA test designed to detect neural tissue emboli in blood (Anil et al., 1999; Love et al., 2000).

### 1.7.4.2 Glial fibrillary acidic protein (GFAP)

GFAP is already well documented as a marker for acute astrocyte injury (Van Geel et al., 2002; Missler et al., 1999). It is a non-soluble, acidic, cytoskeletal monomeric protein molecule with a molecular mass of 40 to 53DA and an iso-electric point between 5.7 and 5.8 (Rueger et al., 1981). GFAP has previously been measured in CSF and in blood as an indicator of CNS pathology (Missler et al., 1999). GFAP belongs to the class-111 intermediate filament proteins and consists of 432 amino-acids (Reeves et al., 1989). Intermediate filaments are filamentous developmentally regulated structures 10-nm in diameter that forms part of the cytoskeleton of most mammalian cells (Kelley et al., 2000). Cytoskeletal GFAP is packed into polymers (Petzold et al., 2004), after break-up of the GFAP polymer following injury a soluble fragment of GFAP of approximately 41kDA is released into adjacent fluid compartments (Eng & Ghirnikar, 1994; Petzold et al., 2004). GFAP is present in both white and grey brain CNS tissues and is strongly upregulated during astrogliosis (van Geel et al., 2002). Interestingly much stronger staining for GFAP was detected in BSE positive cow brains as compared in negative controls (Miyashita, Stierstorfer & Schmahl, 2004). GFAP has previously been used as a sensitive marker for the presence of CNS tissue in meat products (Kelley et al., 2000; Schmidt et al., 1999) and in carcass swab samples (Helps et al., 2002). Although previously described as specific for CNS tissue (Eng, Ghirnikar & Lee, 2000), other peripheral tissues are known to demonstrate immunoreactivity to GFAP (Kelley et al., 2000; Schmidt et al., 1999[a]). Nevertheless, the comparatively weak immunoreactivity in these peripheral tissues allows differentiation with tissues of CNS origin (Schmidt et al., 1999[a]).

### 1.7.5 Combined microscopy and immunocytochemistry

Although helpful in identifying suspected brain tissue emboli the use of microscopy alone often produces inconclusive results, further staining of such specimens by the use of immunohistochemical methods has been used to confirm the CNS origin of tissue sections (Davis et al., 1991; Love et al., 2000). Love et al., (2000) demonstrated that in prepared agarose blocks of plasma, buffy coat and red blood cell fractions, the brain tissue fragments in the samples were exclusively found in the buffy coat fractions of the blood samples. In addition it was found that the Cytoblock preparations provided greater concentration of the buffy coat fraction than did the agarose blocks. Larger fragments of brain tissue were visible by conventional microscopic examination even in those samples containing only 50-mg of brain tissue per 100-ml. Trial immuno-staining with antibodies to NSE, MAP-2, neurofilament proteins, synaptophysin, S-100ß and GFAP showed labelling of brain tissue and no reaction to bone marrow, skin or muscle. The strongest and most consistent staining of both grey and white matter was achieved with the use of anti- S-100ß antibody staining. (Love et al, 2000).

### 1.7.6 Brain tissue emboli and coagulation abnormalities

Severe head trauma and pulmonary embolism of brain tissue is known to be associated with changes in coagulation parameters, including that of prothrombin time (PT) (Ogilvy et al., 1988; Anil, Krailadsiri & Seghatchian, 2002). In previous studies between 3 and 64% of traumatic head injury patients were reported to develop a coagulopathy soon after admission to hospital (Kuo, Chou, & Chio, 2004). The mechanism is believed to involve release of tissue factor (thromboplastin) from damaged tissue, which initiates activation of the clotting cascade via the extrinsic pathway. When any tissue is damaged, tissue factor is released from vascular compartments and is also synthesised by some cells including monocytes in response to inflammatory changes, which follow an injury (Utter et al., 2002). Brain parenchyma and meninges contain high levels of thromboplastin and other systemic coagulation components and it is suggested that brain tissue emboli may result in the release of high concentrations of tissue factor into the circulation, which may trigger the coagulation cascade (Simpson, Speed & Blumbergs, 1991). Reports in the medical literature support

a correlation between coagulation abnormalities and brain tissue destruction in contrast to brain compression alone, and suggest that measurement of coagulation parameters may be diagnostic and prognostic for some cases of head injury (van der Sande et al., 1978). Prothrombin time (PT) has previously been investigated in plasma from cattle in which brain tissue embolism had occurred after stunning, with inconclusive results (Anil, Krailadsiri & Seghatchian, 2002). An experimental study using rabbits investigated the response to injection of brain tissue thromboplastin and found an immediate effect of prolonged PT time due to activation of the clotting cascade by thromboplastin (Tvedskov et al., 1996). The release of brain tissue thromboplastin in cattle and sheep after stunning and in the presence or absence of brain tissue emboli may be a useful marker test for detection of carcasses in which brain tissue contamination has occurred.

### 1.7.7 Use of the polymerase chain reaction for the detection of CNS tissue fragments in the carcass

#### 1.7.7.1 Conventional PCR

The development of the polymerase chain reaction along with the availability of *Taq* DNA polymerases (Saiki et al., 1985) has made it possible to amplify selected regions of the genome many fold to levels at which detection becomes possible. The application of PCR provides the potential for accurate and sensitive techniques for the detection of CNS tissues in both meat and meat products and in ruminant feeds that has not previously been attainable by other methods (Schmidt et al., 1999[a]; Love at al., 2000).

An immuno-quantitative PCR has been described for the detection of CNS tissues using an ELISA for the immuno-detection of bovine brain PrP in combination with real-time PCR to amplify the biotinylated reporter DNA (Gofflot et al., UNPUBLISHED). In a separate study, a PCR assay has been developed based on the amplification of a fragment of 16S rRNA gene of mitochondrial DNA that has allowed the detection of vertebrate tissues in feedstuffs down to a detection limit of 0.0625% (Bottero et al., 2003). A PCR assay has been developed to detect glial fibrillary acidic protein (GFAP) mRNA of bovine CNS tissue in meat and meat products. Although GFAP mRNA is

present in small amounts in peripheral tissues the study reports a technique of heat inactivation of non-CNS signals prior to RNA extraction (Seyboldt et al., 2002).

### 1.7.7.2 Real-time PCR

Conventional PCR methods can be used to detect a DNA template in a sample, but for the accurate quantification of the amount of template DNA present, real-time PCR must be used. The use of probes linked to fluorescent makers allows detection of the amplified DNA as it forms (Bustin, 2000). Fluorescence values are automatically recorded during every amplification cycle and represent the amount of product DNA formed. Larger amounts of starting template DNA will require fewer amplification cycles to reach a statistically significant quantity above that of background levels. This point is called the threshold cycle ($C_t$-cycle) and occurs during the exponential phase of amplification (Bustin, 2000). The Ct value can be translated into a quantitative result by comparison with a standard curve.

A real time quantitative PCR assay based on the amplification of the sex-determining region of the bovine Y chromosome (SRY) along with normalisation for bovine 28S rDNA has previously been used to quantify the contamination of carcasses as a result of the practice of carcass splitting (Helps et al., 2004).

## 1.8 POTENTIAL ROUTES OF DRAINAGE OF BRAIN TISSUE FRAGMENTS FROM THE HEAD

### 1.8.1 Venous Drainage of the head

The cranial system of venous sinuses is responsible for drainage of blood from the brain. All venous sinuses are external to the dura mater and in the head lie between the dura mater and the periosteum, in the spine these sinuses are found in the epidural space.

At the foramen magnum there is continuity of the cranial sinuses with the longitudinal sinuses of the spine. The venous sinuses are devoid of valves both in the brain and also throughout the length of the spine. This feature may allow bacteria or tumour cells to migrate from abdominal organs directly to the brain (Batson, 1940). It is possible that emboli may also travel from the brain to other areas of the body by this route.
The cerebral sinuses are grouped into dorsal and basilar (ventral) systems. The smooth walled dorsal sagittal sinus lies in the base of the falx cerebri in the median plane. It divides into the paired transverse sinus which itself gives off rostral and caudal branches. The rostral branch becomes the temporal sinus and forms the basilar system of venous sinuses. The caudal branch forms the condylar sinus and makes a smooth transition along with the basilar sinus into the internal vertebral plexus of the spine.

This system consists of two pairs of longitudinal vessels lying within the epidural space and continuing along the length of the canal. Segmental anastomoses between these vessels give a ladder-like appearance in which the spaces are taken up by the intervertebral discs, which bulge into the canal (Batson, 1957). The venous drainage of the vertebral column of the ox was well described by Smuts (1977) and in the sheep by Rauhut (1962) and demonstrates that although both are essentially similar some anatomical differences exist. In contrast to the ox, there are no dilatations of the venous sinuses in sheep and goats and they possess a smooth wall (Ghoshal Koch & Popesko, 1981).

### 1.8.2 Haemodynamics of venous drainage

The main drainage vessels of the head are undeniably the jugular veins. However, since the blood flow in the vertebral veins was first investigated (Batson, 1940), it was discovered that posture may influence which venous system carries the larger quantity of blood. The anatomy of the human internal vertebral plexus has been well described (Groen et al., 1997), and a postural dependency of cerebral outflow has been demonstrated (Valdueza et al., 2000). These studies demonstrated that in the upright position there was collapse of the jugular veins and an increase in the venous outflow by the vertebral system of veins. The vertebral system of veins are protected from the effects of gravity by the bony casing of the vertebrae which provide a non-collapsible conduit for venous return that is capable of supporting a gravitational pressure gradient parallel to that of the arteries (Zippel, Lillywhite & Mladinich, 2001). The result of this arrangement is that in a prone position the jugular veins are the principal drainage vessels of the head, in an upright posture however, the jugular veins undergo complete or partial collapse and the vertebral veins become the major drainage vessels of the head. In the head down position the system is believed to function in reverse, with blood from the posterior extremities being shunted first into the plexus and then into the anterior vena cava prior to return to the heart. The distended dural sac at the dependant end occludes the plexus so that only one end of the plexus is to be functional at any given time (Dilenge & Perey, 1973). The effect of a head down position on blood flow has been investigated in snakes using fluoroscopic methods. In these studies conducted on one snake no venous return was observed in the vertebral plexus while at the same time the jugular veins became engorged (Zippel, Lillywhite & Mladinich, 2001). A similar posture dependant division of blood flow was observed in monkeys (Epstein et al., 1970).

The effect of posture on blood flow from the head is important in stunning and slaughter of sheep and cattle as the effects of hoisting the carcass after stunning for sticking and exsanguination may affect the drainage of blood from the head. If such a change in venous drainage were to occur, the dissemination of neural emboli in the circulation might also be affected.

### 1.8.3 Lymphatic drainage of the head

Following the use of captive bolt guns extensive haemorrhage and tissue damage are evident on examination of the brain. The dural membrane is invariably ruptured especially after the use of a penetrative CBG. In a recent study of cattle brains after CBG stunning extensive subarachnoid haemorrhage was observed in nearly all cases (Farag, 2002).

The dura mater is the thick outer membrane of the brain and is fused with the inner periosteum of the skull. The cranial venous sinuses are enclosed within the thickness of the dura mater. The dura forms many folds, one of which extends into the division between the cerebral hemispheres and is referred to as the falx cerebri. Below the dura mater is the more delicate arachnoid mater. The space below the arachnoid mater is called the subarachnoid space and is composed of numerous trabeculae and filaments. It is within this space that the cerebrospinal fluid (CSF) is found.

The CSF is largely produced by the lining of the ventricular system within the brain. This fluid is drained from the subarachnoid space partly through the arachnoid villi or granulations that project through the arachnoid mater into the dorsal sagittal sinus of the brain. In this manner some of the CSF is returned to the venous circulation.

A portion of the CSF is drained to the lymphatics of the olfactory region through the cribiform plate. This lymphatic fluid is drained by ducts to the system of cervical lymph nodes before returning to the blood via the anterior vena cava. The lymphatic system is well suited for the transport of particles such as microorganisms and emboli. Studies of lymphatic drainage of the brain of rats using Indian ink have demonstrated drainage to cervical lymph nodes within 30 minutes (Kida, Pantazis & Weller, 1993). Similar findings have been observed in rabbits and cats (Bradbury & Cole, 1980). Such particles become trapped in the nodes and are removed from the lymph fluid. Studies have demonstrated increased drainage of CSF via the lymphatic system following raised intra-cranial pressure (Caversaccio, Peschel & Arnold, 1996) such as might occur immediately following the use of captive bolt gun stunning.

After CBG stunning fragments of brain and blood from ruptured vessels will be mixed with CSF in the sub-arachnoid space. It is likely that some of this material will be drained into the dorsal sagittal sinus by passage through the arachnoid villi. Such material on entering the venous system might be drained from the head via the jugular veins or perhaps by the internal vertebral plexus of the spine.

It is possible that like the CSF a portion of this material might enter the lymphatic system and be drained to the lymph nodes of the neck. The deep cervical lymph nodes are located within the muscles of the neck and are not classified as SRM. If neural emboli were to be trapped by such nodes it is possible that contamination of edible tissues with neural tissue might occur. It is likely that the lymphatic system plays a secondary role to that of the venous system in transport of neural emboli but might still carry a significant risk to the consumer of meat.

## 1.9 REDUCTION OF CNS TISSUE CONTAMINATION OF THE CARCASS

The recognition that current stunning and slaughter practices can result in contamination of carcasses with material classified as SRM, has given rise to suggestions for preventive measures. Abandoning the use of stunning would have severe welfare consequences and is not in-line with UK legislation concerning the slaughter of animals. Alternative stunning methods for cattle have been suggested including modifications to current methods, however no commercially viable method is presently available.

Suggestions for preventing contamination as a result of carcass splitting include the use of hot-boning in which the carcasses are butchered while still warm. This method already used in some countries affords the advantage of avoiding carcass splitting and the consequent risk of CNS tissue contamination. In the UK the recent development of an oval saw that removes the vertebral column in its entirety including the dorsal root ganglia has offered an alternative solution to the problem of carcass splitting. This saw is currently under-going trials to assess its commercial viability (Remcolm project, Silsoe Research Institute, Bedford, UK).

## 1.10 AIMS OF THE STUDY

By the use of a variety of applied methods, the experiments described in this thesis have investigated the potential of current stunning methods used on cattle and sheep to cause introduction of brain tissues into the venous circulation. Furthermore, the risk of such brain tissue emboli contaminating edible parts of the carcass with tissues of potentially high BSE infectivity has been explored.

The specific questions this study has attempted to answer are given below:

1. Which stunning methods currently in use/or available in the UK carry a risk of brain tissue embolism?
2. What is the frequency of brain tissue entry into the venous circulation after stunning by captive bolt gun in cattle and sheep?
3. Can brain tissue fragments and proteins disrupted by captive bolt gun stunning of cattle and sheep penetrate the pulmonary capillary filter and enter the arterial circulation within the time-frame of commercial stunning and slaughter?
4. How much brain material enters the venous blood of sheep after captive bolt gun stunning?
5. What are the potential venous drainage routes of emboli from the head following the use of captive bolt gun stunning?
6. What organs of the carcass may be contaminated with brain tissue emboli after stunning?

# CHAPTER 2 MATERIALS AND METHODS

## 2.0 INTRODUCTION

The general methodologies employed in the experiments in this thesis are described in this chapter. More specific descriptions are included with the results and discussion of each experiment. Experiments were carried out in cattle at a local abattoir reserved for slaughter of cattle over thirty months of age (Jarrett's and Sons Abattoir, Oldland Common, Bristol, UK). All experiments conducted in sheep took place at the Langford site of the Bristol School of Veterinary Science.

## 2.1 DETECTION OF BRAIN TISSUE CONTAMINATION IN BLOOD AND OTHER TISSUES USING ELISA AND A COMBINATION OF MICROSCOPY AND IMMUNOCYTOCHEMISTRY

### 2.1.1 Aims

- To determine the frequency of brain tissue embolism after captive bolt gun stunning in cattle and sheep.
- To investigate the potential for brain tissue fragments to penetrate the lungs and enter the aortic circulation.
- To investigate the potential of electrical stunning to cause entry of brain tissue fragments into the venous circulation.
- To investigate the potential for organ contamination with brain tissue fragments.

### 2.1.2 Principles

Immunoassays are based on the specificity of the reaction possible between antibodies and antigens. The high level of sensitivity achievable with these assays allows the accurate detection and quantification of the analyte of interest.

### 2.1.3 Experimental design

The sample size of 100 animals in each test group was chosen based upon the estimated design sensitivity that would be required to detect and establish the frequency of brain tissue embolism following the use of each captive bolt gun. These estimates were made based upon the results of similar investigations of brain tissue embolism after stunning previously conducted on small groups of animals (Anil et al., 1999; 2001). The number of sample animals chosen in these studies was also constrained by logistical and cost considerations of sampling from large numbers of cattle and sheep.

### 2.1.4 Anaesthetic methods

Anaesthetic drugs and regimens were employed in many of this series of experiments in both cattle and sheep. Standard veterinary methods of anaesthetic monitoring were used. The regimen used in both species is given below.

### 2.1.4.1 Induction mixture

2% xylazine (0.4mg/kg) (Bayer, Bury St. Edmunds, UK) and 10% ketamine (12mg/kg) (Fort Dodge, Southampton, UK) were administered together in the same syringe.

### 2.1.4.2 Maintenance mixture

A mixture of guaiphenesin, ketamine and xylazine (100:6:3) was prepared by addition of 30-ml of ketamine and 15-ml of xylazine to a 500-ml bottle of guaiphenesin (10% guaiphenesin (Chassot Ltd, Preswick, UK), 10% ketamine and 2% xylazine). This mixture forms a precipitate within 2 to 3 days of preparation so warming and agitation was necessary to re-dissolve all constituents before use.

### 2.1.4.3 Euthanasia drugs

In some instances euthanasia was performed by intravenous administration of barbiturate solution (Euthatal (200mg/ml), Bayer, UK).

### 2.1.4.4 Administration of anaesthetic to cattle

All anaesthetic preparations were injected intravenously through a pre-placed catheter inserted into a jugular vein.

### 2.1.4.5 Administration of anaesthetic to sheep

The anaesthetic induction mixture was administered by an intra-muscular injection into the caudal thigh area of each animal. The maintenance mixture was given by intra-venous injection via a pre-placed catheter inserted into the jugular vein.

#### 2.1.4.6 Monitoring of anaesthetic depth

Conventional methods of checking anaesthetic depth in all animal subjects were used including respiratory rate and depth, heart rate, palpebral and corneal reflexes and withdrawal reflexes. More anaesthetic mixture was administered as necessary.

### 2.1.5 Surgical procedures

#### 2.1.5.1 Implantation of jugular catheters in cattle for administration of anaesthetic

It was necessary to implant indwelling catheters into the jugular veins to allow administration of anaesthetic to the cattle without undue interruption to the normal running of the abattoir. Animals were first restrained in a crush in the lairage area of the abattoir and a twelve gauge 52-mm intravenous angiocatheter (Intraflon, Vycon Ltd, Ecouen, France) was introduced in a caudal direction into the jugular vein. A steady and strong flow of blood from the end of the catheter indicated correct placement within the lumen of the vessel. A bevelled polyvinyl catheter (1.5-mm diameter lumen, Klarer PVC, UK) was then threaded into the jugular vein using the pre-placed angio-catheter to allow access to the lumen of the jugular vein. When at least 12-cm of PVC catheter was advanced into the vessel the guiding catheter was removed and the catheter was secured in place using both tape and super-glue (RS Ltd. UK). The PVC catheter was immediately flushed with 5% heparinised saline (Multiparin 5000 iu/ml, UK) solution to prevent clotting within the tubing and then capped to prevent leakage of blood. The catheter was further protected by more adhesive tape placed around the neck of the animal. The cattle were then moved through one at a time to the stunning box where the injection port of the jugular catheter was retrieved and the anaesthetic induction mixture was administered as a bolus injection of 15-ml of 10% ketamine and 6-ml of 2% xylazine. Immediately as anaesthesia was induced the animal was released on to the killing floor of the abattoir by release of the gate of the stunning box. At this point a forty-ml dose of maintenance anaesthetic mixture was given by way of the jugular catheter portal.

### 2.1.5.2 Implantation of jugular catheters in sheep for administration of anaesthetic

The anaesthetic induction mixture was administered by an intra-muscular injection into the caudal thigh area of each animal (12mg/ml ketamine and 0.4mg/kg xylazine). Allowing at least five minutes for induction of anaesthesia each sheep was transferred to the procedure laboratory and placed in a hammock lying on the sternum. To allow further administration of anaesthetic a 22-g, 1-inch intravenous catheter (Angiocath, Becton Dickinson Infusion Therapy Systems Inc., Utah, USA) was introduced into the jugular vein. The jugular catheter was secured in place by way of tape and flushed with heparinised saline to prevent blockage with clots. Anaesthetic depth in each animal was maintained by administration of between 5 and 20-ml of anaesthetic maintenance mixture.

### 2.1.5.3 Implantation of jugular collection catheters

When a satisfactory level of anaesthesia was achieved and the animal was lying on the right flank and employing a 'cut-down' method, a 14-g Foley balloon catheter (Benkat instruments, UK) in cattle and 2-mm polyvinyl catheters (Klarer PVC, UK) in sheep was advanced in a cranial direction into the jugular vein lumen and secured in place with string ligatures. The balloon cuff of the Foley catheter implanted in cattle was also maximally inflated to prevent any leakage of blood flow around the catheter. A similar procedure was performed on the contra-lateral jugular vein after turning the animal onto the left flank. When both jugular veins were cannulated a plastic Y-piece was attached to each catheter joining to a single length of plastic tubing to allow collection from a single catheter end.

### 2.1.5.4 Implantation of aortic collection catheters

Arterial catheters were used in both cattle and sheep to allow sampling of aortic blood. Anaesthesia was induced and maintained and the animals were placed on the left side. An incision was made over the jugular groove to expose the jugular vein. Using blunt dissection the under-lying carotid artery was located and identified by palpation of a strong pulse. The artery was then partially freed from the connective tissues and two

string ligatures were placed around the vessel. Using these ligatures to limit blood loss a small V-shaped incision was made in the vessel. A bevelled polyvinyl catheter of up to 1-cm diameter in cattle (Klarer PVC, UK) and 2-mm in sheep was introduced into the carotid artery and advanced into the proximal aorta, as determined by measurement of the entry point of the catheter to the estimated position of the heart on the skin surface. An average length of approximately 30 and 90-cm of catheter was inserted into the carotid arteries of sheep and cattle respectively. The catheter was then secured in placed with the string ligatures and the end plugged with a cap.

### 2.1.6 Preparation and administration of brain tissue suspensions

#### 2.1.6.1 Brain tissue suspensions

The procedure used to prepare suspensions was designed to mimic the nature of brain tissue fragments dislodged during normal captive bolt stunning and that might enter the circulation. Loose brain tissue created by previous stunning of an animal with a penetrating captive bolt gun during the slaughter process was carefully removed by flushing with normal saline followed by aspiration with a 10-ml syringe through the bolt hole of a fresh carcass. The tissue was suspended by vigorous agitation in normal saline in a ratio of 1-g of tissue to 9-ml of saline. A 10-ml sample of suspension therefore contained approximately 1-g of brain tissue. Previous studies suggest that this is a conservative estimate of the amount of brain tissue that may be dislodged from the cranial cavity during stunning with a captive bolt gun (Anil et al., 2002). The aim in obtaining and suspending the brain tissue in the manner described was to achieve a comparable total volume and range of particle sizes to the embolic showers produced as a consequence of stunning with a captive bolt gun.

2.1.6.2 Administration routes of brain tissue suspensions

*2.1.6.2.1 Jugular vein injection*

Brain tissue suspensions were administered as bolus injections in the jugular veins by way of pre-placed catheters in most cases. In some experiments injections were made directly into the jugular vein lumen by way of a syringe and needle.

*2.1.6.2.2 Angularis oculi vein injection*

The area ventral and rostral to the medial canthus of the eyes was clipped and cleaned. Using digital pressure, the angularis oculi vein was raised to facilitate catheterisation. A cut-down technique was used in combination with digitally applied tension on the vein to allow a 22-g, 1-inch angiocatheter to be introduced to the lumen of the vein. Withdrawal of blood confirmed correct placement of the catheter within the vein. A 1-ml quantity of saline and heparin solution was used to flush the catheter to prevent clotting within the lumen. The catheter was secured with tape. Brain suspensions were injected into each vein using the pre-placed catheters and a 10-ml syringe.

*2.1.62.3 Intra-cranial injection*

Injections into the cranium were achieved by way of a pre-drilled hole in the midline of the skull or by way of a bolt-hole created by prior stunning using a penetrating captive bolt gun. A 10-ml syringe with a 2-inch 16-g needle was used to inject the brain suspension into the cranial cavity.

### 2.1.7 Pre-slaughter stunning

#### 2.1.7.1 Placement of animals for stunning

##### *2.1.7.1.1 Cattle*

Cattle were stunned on the killing floor of the abattoir while anaesthetised and in left lateral recumbency.

##### *2.1.7.1.2 Sheep*

Sheep were stunned while anaesthetised and in lateral recumbency on a table with the head of the animal extended over the table edge.

#### 2.1.7.2 Captive bolt guns

##### *2.1.7.2.1 Penetrating cartridge activated captive bolt gun (cattle)*

Cowpuncher Cash 8000 Model Stunner (Accles & Shelvoke, Birmingham, UK)
A 3-grain cartridge was routinely used throughout the project.

##### *2.1.7.2.2 Non-penetrating cartridge-activated captive bolt gun (cattle)*

Cash Magnum Knocker Concussion Stunner (Accles & Shelvoke, Birmingham, UK)
A six- grain cartridge was routinely used throughout the project.

Figure 2.1 Photograph of the penetrating captive bolt gun (Cash Magnum Knocker, Accles & Shelvoke, Birmingham, UK) (right) and the non-penetrating captive bolt gun (Cowpuncher Cash 8000 Model Stunner, Accles and Shelvoke, Birmingham, UK) (left)

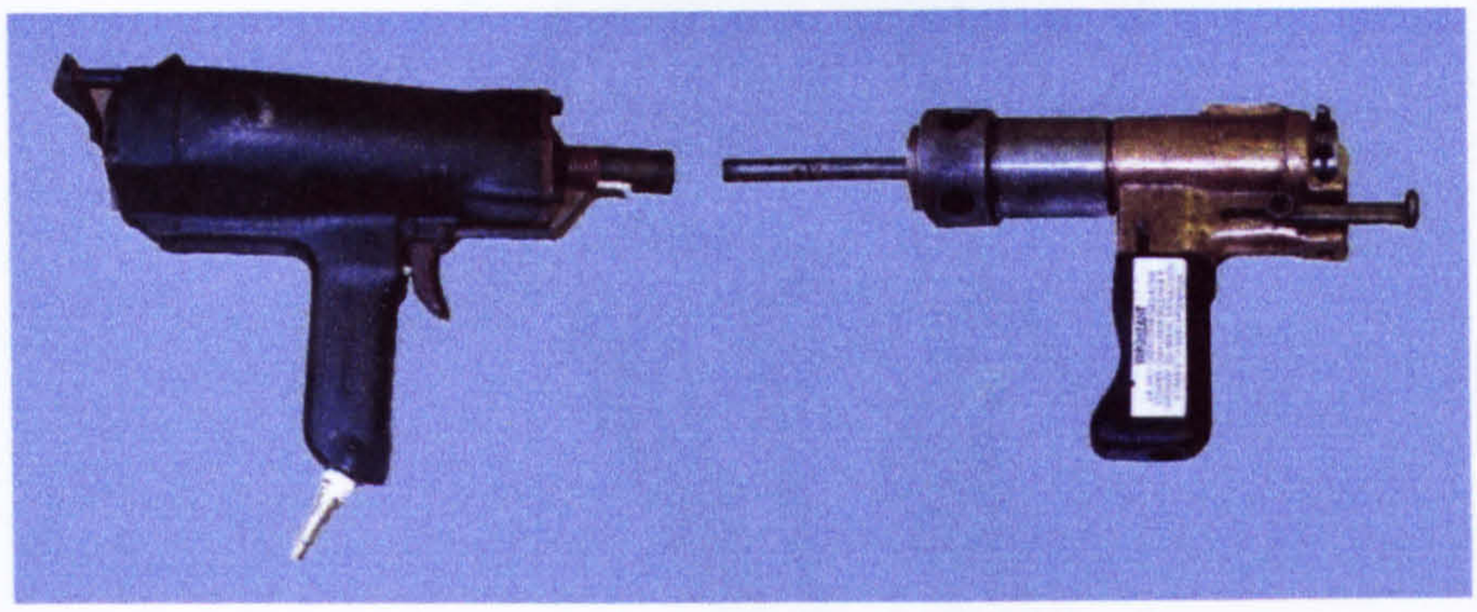
*2.1.7.2.3 Penetrating cartridge activated captive bolt gun (sheep)*

A Temple Cox Mark 10 humane stunner (Accles & Shelvoke, Birmingham, UK) with 1.25-grain cartridges was used to stun sheep through out the project.

*2.1.7.2.4 Penetrating pneumatically activated captive bolt gun (sheep)*

A Cash Ramrod pneumatic stunner (Accles & Shelvoke, Birmingham, UK) without air-injection facility was used to stun sheep. A cylinder of compressed air at 100 psi was used to activate the device.

Figure 2.2 Photograph of the penetrating pneumatically-activated captive bolt gun (Cash Ramrod pneumatic stunner, Accles & Shelvoke, Birmingham, UK) (left) and the cartridge-activated penetrating captive bolt gun (Temple Cox MK. 10 humane stunner, Accles & Shelvoke, Birmingham, UK) (right)



2.1.7.3 Stunning position

2.1.7.3.1 ***Cattle***

*2.1.7.3.1.1 Penetrating captive bolt gun*

The muzzle of the CBG was placed at right angles to the skull at the intersection of two imaginary lines drawn from the eyes to the base of the contra-lateral horn

*2.1.7.3.1.2 Non-penetrating captive bolt gun*

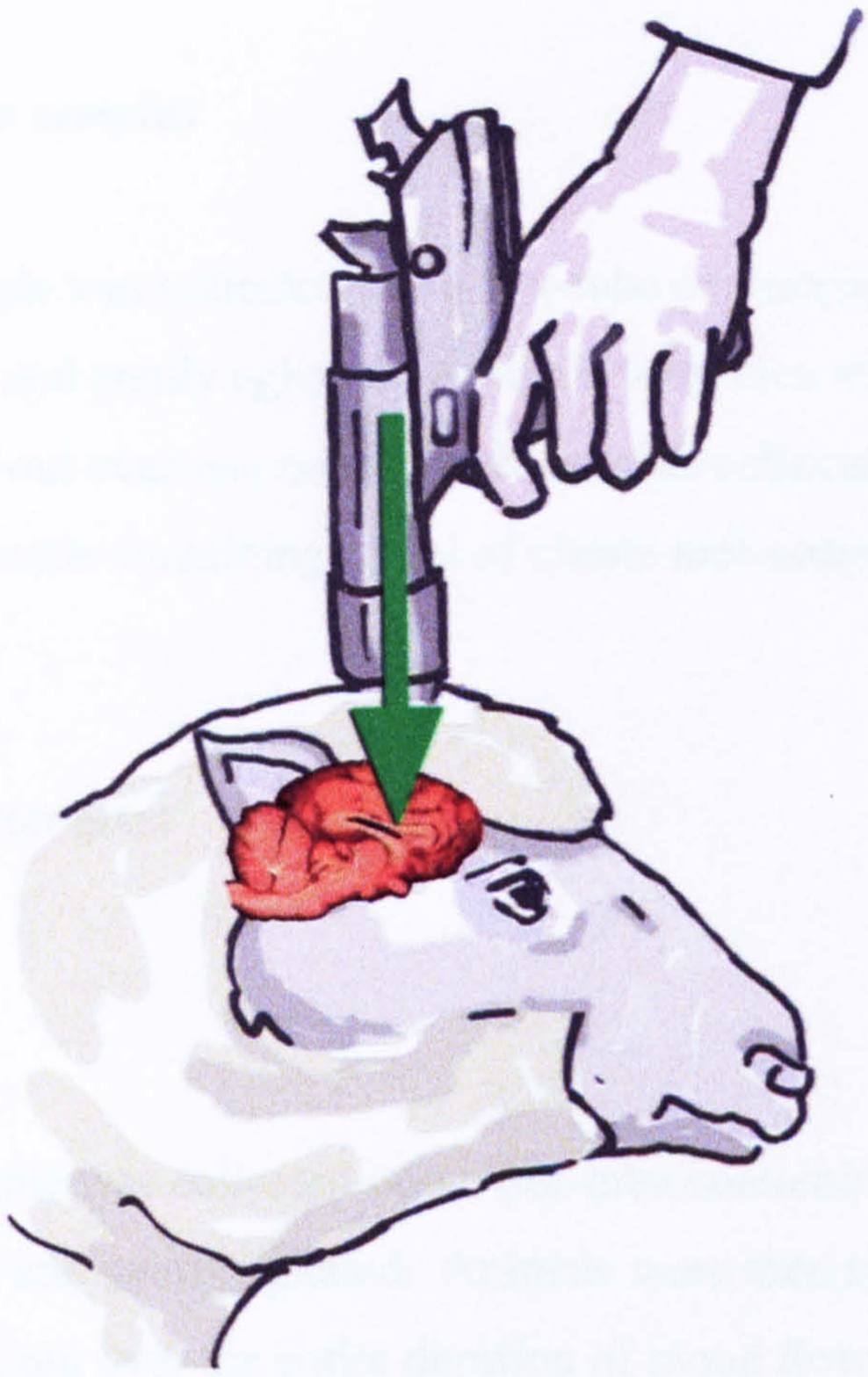
The muzzle was placed approximately 20-mm above the point indicated for use with a penetrating captive bolt gun before activation for stunning.

Figure 2.3 Ideal stunning position with non-penetrating (blue dot) and penetrating (red dot) captive bolt guns for use on cattle

2.1.7.3.2 ***Sheep***

All sheep were stunned by an experienced slaughter-man using standard commercial techniques. Briefly, the muzzle of the CBG was placed on the highest point of the head, on the midline of the skull aiming straight down before activation of the gun

Figure 2.4 Ideal stunning position with a penetrating captive bolt gun for use on polled sheep



2.1.7.4 Re-stunning

Animals that were judged to be inadequately stunned by the initial shot were immediately re-stunned by a second stun placed close to but away from the immediate area of the original stunning position. Conventional methods of estimating recovery after stunning including observation of rhythmic breathing and return of eye reflexes were used to detect animals that were poorly stunned after the initial shot.

### 2.1.8 Sample collection

#### 2.1.8.1 Blood

##### *2.1.8.1.1 Jugular samples*

A 10-ml pre-stun sample was collected into a test-tube containing citrate anti-coagulant (3.8% citrate, pH 5.2) and gently agitated. Animals were then stunned and blood collection was carried out over one minute with samples collected every ten seconds into a 250-ml plastic bottle containing 25-ml of citrate anti-coagulant.

##### *2.1.8.1.2 Aortic samples*

###### *2.1.8.1.2.1 Sheep*

A 10-ml pre-stun sample was collected into a test-tube containing citrate anti-coagulant (3.8% citrate, pH 5.2) and gently agitated. Animals were then stunned and blood collection was carried out over the entire duration of blood flow with samples collected every minute into a 250-ml plastic bottle containing 25-ml of citrate anti-coagulant.

###### *2.1.8.1.2.2 Cattle*

A 10-ml pre-stun sample was collected into a test-tube containing citrate anti-coagulant (3.8% citrate, pH 5.2) and gently agitated. Animals were then stunned and blood collection was carried out over two minutes with samples collected every ten seconds into a 250-ml plastic bottle containing 25-ml of citrate anti-coagulant.

##### 2.1.8.1.3 *Exsanguinated blood*

Approximately five hundred millilitres of exsanguinated blood was collected as rapidly as possible following electrical stunning and sticking by holding a 500-ml plastic container with anticoagulant in the blood flow from the stick wound.

#### 2.1.8.2 *Organs*

Organ samples including the spleen, liver and kidneys were taken at post-mortem via a mid-line abdominal incision. Organs were removed in their entirety and placed in sterile, labelled bags.

### 2.1.9 Slaughter

After sample collection, animals were exsanguinated in the normal commercial manner by severing the main vessels of the neck. In some cases animals were killed by intra-venous administration of barbiturate.

### 2.1.10 Macroscopic brain examination

At a commercial abattoir (St. Merryn Meat Ltd, Merthyr Tydfil, Wales) in which a fully automated electrical stunning device (Jarvis, New Zealand) is used, fifteen fresh cattle heads were removed from the line immediately following slaughter, exsanguination and subsequent decapitation.

The dorsal cranium was removed by the use of a saw to expose the dorsal surface of the brain in each case. The dura was reflected and the surface of the brain cortex was examined for macroscopic evidence of trauma or haemorrhage. The specimens were labelled and photographs taken in every case. The brain was then carefully removed from the cranium and the ventral surface examined and photographed. Each brain was also weighed to give an average weight of brains examined in the study. Sagittal and coronal sections were made of each brain and the internal structures examined.

### 2.1.11 Laboratory processing of samples

Samples of blood and tissues were taken from both sheep and cattle for processing and analysis by the methods described below. These experiments formed part of the larger study of contamination of the carcass after stunning. Samples were examined for the

presence of brain tissue fragments by microscopy and immunocytochemistry and for the presence of elevated levels of GFAP by ELISA.

### 2.1.11.1 Separation of blood sample fractions

Two 1-ml aliquots of the whole blood samples were removed from each sample and rapid frozen in liquid nitrogen before transfer to a freezer at -30°C for storage. The remaining blood in the collection bottles were placed in a pre-chilled centrifuge (MSE, UK) (5°C) and spun for thirty minutes at *800 x g*. From each fractionated sample two, 1-ml aliquots of the plasma supernatant were drawn off by pipette and stored at -30°C. The remaining plasma overlying the buffy coat layer as well as the greater quantity of the under-lying erythrocyte fraction was suctioned off using a peristaltic pump (603u, Watson-Marlow Ltd, Falmouth, England). The remaining buffy coat was then divided into two samples. One of these samples was combined with an equal volume of twenty percent formalin in a Universal plastic tube and sent for immunocytochemical testing. The remaining buffy coat was placed in three 1-ml sample tubes and rapid frozen in liquid Nitrogen. Any remaining buffy coat was retained. All samples to be stored were placed in labelled plastic bags and transferred to a freezer at -30°C.

### 2.1.11.2 Samples sent for analysis

Samples of buffy coat were analysed by ELISA and by microscopy and immunocytochemistry. All samples collected including those taken immediately prior to stunning were analysed by GFAP ELISA. Due to financial and time constraints only a proportion of samples were analysed by microscopy and immunocytochemistry. Initially these samples included only those taken between ten and thirty seconds after stunning as previous work had indicated that the majority of positive samples were taken during this period. Later in the project the number of samples from each animal analysed by this technique was increased to include samples taken between zero and forty seconds. In addition, samples that appeared to be clotted during processing were sent as additional samples for processing by this technique.

### 2.1.11.3 Labelling and storage of samples

#### *2.1.11.3.1 Cattle jugular samples*

All storage bottles for the various fractions to be removed from the samples were previously labelled with a capital C for cattle and secondly with the individual sample number that remained consistent throughout all stages of sampling and processing. The one millilitre sample bottles were capped with transparent caps to allow easy identification of cattle samples.

#### *2.1.11.3.2 Cattle aortic samples*

These samples were labelled with a capital C for cattle followed by a letter indicating the animal designation finally all samples were consecutively numbered.

#### *2.1.11.3.3 Sheep jugular samples*

Sheep samples were labelled 'SH' followed by the sample number. Green caps were used to cap sample tubes that were placed in labelled bags and stored in a freezer designated for sheep samples.

#### *2.1.11.3.5 Sheep aortic samples*

These samples were labelled with a capital S for sheep followed by a letter indicating the animal designation finally all samples were consecutively numbered.

#### *2.1.11.3.6 Sheep organ samples*

Organ samples were labelled with a capital S for sheep followed by a letter designating the experiment that included V for the investigation of drainage to organs by way of the vertebral plexus and F for the filtration trials. A further number was given to each individual sample.

### 2.1.12 Analytic methods

#### 2.1.12.1 Enzyme Linked Immunosorbent Assay (ELISA)

##### *2.1.12.1.1 Principles*

All immunoassays involve the use of antibodies as reagents. Enzyme immunoassays use enzymes linked to an antibody in the assay to allow quantification of an unknown reactant by way of a colour change. The ELISA used in this experiment is classified as an indirect sandwich ELISA. In these assays, antibodies are passively attached to a solid phase (ELISA plate) and specific antigens in the sample are captured. An additional detecting antibody is then added followed by the addition of an anti-species enzyme conjugate. A substrate is then added to produce the colour change that is quantified against a dilution curve of a standard in a spectrophotometer.

##### *2.1.12.1.2 Reactants*

- Polyclonal Rabbit Anti-Glial fibrillary acidic protein (DakoCyomation, Glostrup, Denmark)

- Bovine GFAP Standard (American Research Products, USA) reconstituted with 200µl of distilled water to give 1mg/ml solution. Then 5µl aliquots were placed into siliconised eppendorfs and stored at -70°C.

- Monoclonal Mouse anti-GFAP (Chemicon International, California, USA).

- Alkaline phosphotase conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., USA) Reconstituted with 0.5-ml distilled water and stored at -30°C. The diluted conjugate was used on the same day and any remaining reagent was discarded.

- Alkaline phosphotase substrate ( p-Nitrophenylphosphate or pnpp) (Sigma, St. Louis, USA)

#### *2.1.12.1.3 Buffers*

- Carbonate buffer (Sigma-Aldrich, St. Louis, USA)
- Phosphate buffered saline 10x PBS

  Sodium Chloride- 8g

  KH2PO4 0.2-g

  NA2HPO412H2O 143.3g

  KCl 0.2-g
- Polyoxyethylene Sorbitan Monolaurate (Tween 20, Sigma, St. Louis, USA)
- PBS-Tween-milk (0.5ml Tween in 1L PBS)
- Milk Marval dried low fat milk
- PBS-Triton (0.5%) 5ml/L

#### *2.1.12.1.4 Consumables*

- ELISA plates (Greiner bio-one, Glouscester, UK)
- Universal tubes (Bibby-Sterilin,Ltd, Staffordshire, UK)

#### *2.1.12.1.5 Capture ELISA for GFAP*

ELISA plates were coated with polyclonal Rabbit anti-GFAP (DAKO ZO334) and incubated for one hour at 37°C in a humidity chamber. The antibody was prepared by diluting at one part to five hundred in carbonate buffer (Sigma C-3041) and 50 µl was added to each well. The antibody was discarded and the plates were washed for four rinse cycles at 200 µl of PBS- tween20 (0.05%) per well in an automated plate washer. (Labsystems, UK). To prevent non-specific binding of protein, 200µl of PBS-Tween 20/5% non-fat dried skimmed milk was added to each well and incubated for one hour at room temperature. The plates were then washed as previously described. Bovine GFAP (American research products 03-62007) was used as the standard and was prepared by taking 1mg/ml of stock solution and diluting at 1:500 with PBS-Triton-X-100 (0.5%). These samples were aliquoted into 150µl samples in silicone coated eppendorfs and stored at -70° C. For each ELISA plate used the GFAP standard was serially diluted over the range of concentrations from 2000ng/ml to 0.244 ng/ml. On a

separate plate 75 µl of each buffy coat sample was added neat to each well and then 75µl of 0.5%PBS-triton was added to all wells. Using the previously prepared plate with the GFAP standard 50µl of each sample was added in duplicate to a well and the plate incubated at room temperature for ninety minutes. After a further rinse a blocking solution of monoclonal anti-GFAP antibody (Chemicon MAB 3402) diluted 1:10,000 in PBS-tween20/milk is added at 50µl to each well and incubated at room temperature for one hour. The plates were then rinsed. A 50µl volume of alkaline phosphatase conjugated goat anti-mouse IgG antibody (Stratech 715-055-150) was then added to each well after diluting at 1:1000 in PBS/tween20 milk. The plates were incubated at room temperature for one hour. The plates were again rinsed and then 50µl of phosphatase substrate (Sigma 104-105) was added to each well at a concentration of 1mg/ml diluted in carbonate buffer. The plates were then incubated overnight at 4°C to allow development. The following morning the plates were read at a dual wavelength of 405/492nm. The purpose of reading at a dual wavelength was to remove background readings due to properties of the plate which were detected at the higher wavelength of 492nm. A standard curve was generated from the serially diluted standard that allowed quantification of the sample results. Due to the dilution of the samples in PBS-triton a 2x multiplication of the calculated concentration was necessary to obtain the true concentration of the unknowns. At the end of the assay waste wash collected was treated with 1M Sodium Hydroxide and left overnight before being disposal.

#### *2.1.12.1.6 **Controls***

##### *2.1.12.1.6.1 Negative controls*

Pre-stun samples were included on every plate run by ELISA, in addition to the final well in the GFAP standard dilution series in which all reactants were added except for the standard.

##### 2.1.12.1.6.2 *Positive controls*

A known positive sample was added to each ELISA plate in addition to the dilution series of the GFAP standard.

#### *2.1.12.1.7 Repeatability of the assay*

The intra and inter-plate variability was calculated using ten replicates of a dilution of the GFAP standard on separate occasions. The coefficients of variation of the replicate samples from the mean were then calculated.

#### *2.1.12.1.8 Analysis of samples prepared for validation study*

Samples were processed and sent for analysis by GFAP ELISA and by microscopy and immunocytochemistry. Technicians blinded to the identity of samples performed the assays. All samples for GFAP ELISA were repeated in duplicate on the same plate and all positives were repeated on at least one other occasion. The plates were read at 405-nm to obtain the optical density of each well.

#### *2.1.12.1.9 Selection of the positive/negative threshold (cutoff)*

A conservative estimate was used to define a positive sample. It was decided to determine the cut-off value for a positive sample as five times the standard deviation plus the background level. The background was calculated from the mean value of a random selection of thirty pre-stun sample values (assuming a normal distribution of the background OD values obtained by ELISA). [eg. for cattle samples, mean pre-stun OD=0.086 where n=30, SD=0.03, cut off=0.236 (0.086+ 5*0.03)].

#### *2.1.12.1.10 Parallelism*

Equivalent activity between the GFAP standard and the brain tissue in blood suspensions was achieved by plotting the dilution curves for the GFAP standard and for the brain in blood suspensions from the OD values obtained in the assay and from the known dilution factors.

#### *2.1.12.1.11 **Validation***

The sensitivity of the ELISA was assessed by an end-point dilution analysis of prepared brain tissue in blood suspension prepared using both cattle and sheep tissues.

##### *2.1.12.1.11.1 Preparation of brain in blood suspensions*

A small quantity of brain tissue was removed from fresh bovine and ovine carcasses and gently macerated with a mortar and pestle. Then 0.5-g of this tissue was weighed and transferred to a bottle containing 50-ml of fresh anti-coagulated bovine blood. This sample was then mixed by use of a vortex. Five similar brain tissues in blood suspensions were prepared using both bovine and ovine brain tissue and blood.

##### *2.1.12.1.11.2 Dilutions*

Two fold serial dilutions of these preparations were made using an automatic pipette with a sterile tip for each dilution. The final dilution was 0.6µg/ml after 14 further dilutions.

Table 2.1 Concentrations of prepared dilutions of brain tissue in blood

| Dilution | Concentration |
|---|---|
| 1 | 10mg/ml |
| 2 | 5mg/ml |
| 3 | 2.5mg/ml |
| 3 | 1.25mg/ml |
| 5 | 625µg/ml |
| 6 | 312µg/ml |
| 7 | 156µg/ml |
| 8 | 78µg/ml |
| 9 | 39µg/ml |
| 10 | 19µg/ml |
| 11 | 9.8µg/ml |
| 12 | 4.8µg/ml |
| 13 | 2.4µg/ml |
| 14 | 1.2µg/ml |
| 15 | 0.6µg/ml |

*2.1.12.1.11.3 Analysis*

All samples were analysed in duplicate on at least two separate occasions.

*2.1.12.1.11.4* ***Statistical analysis***

A Mann-Whitney 2 sample rank test was performed on the GFAP concentrations detected by ELISA for the positive cattle and sheep samples

A retrospective analysis of the power and sample size based upon the detected difference in frequency was performed for the chosen sample size of 100 and also for larger sample sizes using Minitab version 14.

## 2.1.12.2 Microscopy and immunocytochemistry

### *2.1.12.2.1* ***Principles***

The use of microscopy in the analysis of blood and tissue samples relies upon the experience and judgement of the neuropathologist to correctly identify the prepared tissue sections. Further immunohistochemical staining methods allow confirmation of the identity of tissues initially observed under a light microscope. In this technique antibodies are used to link tissue antigens specifically to a stain that can then be readily identified under a light microscope. The method involves four stages:

- Fixation- Tissues are preserved without disrupting the original structure
- Antigen retrieval- Increase's the reactivity of the antigens that will bind to the detector antibodies.
- Section preparation- Tissue is embedded in paraffin wax and thin sections are made.
- Detection- An enzyme linked antibody is used to produce a colour change following addition of the appropriate substrate.

#### *2.1.12.2.2 Microscopy and immunocytochemistry*

The method for sample processing and analysis has been previously described (Love et al., 2000). In brief, this was as follows. Samples of buffy coat were fixed in formalin, pelleted and embedded in paraffin wax Cytoblocks. 5-µm-thick sections were then cut at multiple levels through the block. Adjacent sections were stained with haematoxylin and eosin or immunostained by a standard streptavidin-biotin-peroxidase method for neurofilament protein (Dako, Ely, UK) or S100β protein (Dako, Ely, UK). Samples were regarded as positive if the Cytoblock sections included fragments of tissue that were immunopositive for both neurofilament protein and S100β protein.

#### 2.1.12.2.3 *Controls*

Negative controls comprised of sections immunostained with an irrelevant antibody or immunostained according to the protocol with the omission of the primary antibody. Other controls included paraffin sections of skin, muscle, bone marrow and brain from slaughtered animals.

#### *2.1.12.2.4 Validation*

The sensitivity of the analytical method was assessed by an end-point dilution analysis of the same prepared brain tissue in blood suspensions prepared for the estimation of the detection limit of the ELISA.

## 2.2 ANATOMICAL INVETSIGATION OF VENOUS CEREBRAL DRAINAGE

### 2.2.1 Aims

- To investigate all venous drainage routes from the head that may contribute towards carriage of brain tissue emboli following the use of captive bolt gun stunning in sheep and cattle.

### 2.2.2 Vascular casting

#### 2.2.2.1 Principles

Injection with a liquid resin that dries and hardens to assume the conformation of the internal lumen of the vessels in to which it is introduced offers an effective method of demonstrating structures that would otherwise be difficult to identify and separate from surrounding soft tissue. The use of concentrated alkaline solutions to macerate all tissues including bone enables the vessel casts to be observed without overlying tissues and reduces the risk of damaging the delicate casts by manual removal of surrounding tissues.

#### 2.2.2.2 Specimen preparation

The animals were anaesthetised by induction with an intra-muscular injection of 10% ketamine (12mg/ml) and 2% xylazine (0.4mg/kg) and were given further doses of pentobarbital (200mg/ml) to ensure deep anaesthesia before being killed by exsanguination by severing the vessels of the lower neck. Prior to exsanguination each animal was injected intravenously with 10,000 IU of heparin to reduce clotting and blockage of small veins. Captive bolt stunning was not used on these animals to limit damage to blood vessels within the head. The heads were removed from carcasses by disarticulation of the fifth and sixth cervical vertebrae.

### 2.2.2.3 Resin preparation

Methyl Methacrylate resin (Tensol 70, Evode Speciality Adhesives, UK) in combination with hardener (Tensol 70, Evode Speciality Products Ltd, UK) was used to prepare the casts. This resin was chosen due to availability, ease of injection due to low viscosity of the resin and the solidity of the cast produced (Mr. S. Gaze, Department of Anatomy, University of Bristol, UK- personal communication). A mixture was used of 1-ml of resin per three drops of hardener along with 1-ml of coloured pigment (Educational and Scientific Products Ltd, UK) for each 10-ml syringe of mixture prepared.

### 2.2.2.4 Injection with methyl methacrylate resin

The external jugular veins of each specimen was identified and partially exposed to reveal the cut ends. Ten millilitres of methyl methacrylate resin (Tensol 70, Evode Speciality Adhesives, UK) was drawn up in a 10-ml syringe and a pigment (red, yellow or blue) (Educational and Scientific Products Ltd, UK) and hardening agent (Tensol 70, Evode Speciality Products Ltd, UK) was added. This resin mixture was then manually injected into both external jugular veins under pressure employing string ligatures around the vessel and syringe tip to prevent leakage. All specimens were left overnight at room temperature to allow hardening of the resin. The following day the heads were skinned and the mandible of each head was removed.

### 2.2.2.5 Maceration of tissues

A 1M (40mg/ml) solution of sodium hydroxide (Prolabo pellets, VWR International, Foutenay sous Bois, France) was prepared and poured into a plastic bucket in which the specimen had been placed such that sufficient solution was added to cover the specimen. The caustic solution at room temperature was refreshed at weekly intervals by the addition of further quantities of sodium hydroxide pellets. The maceration of the specimen was monitored and photographs were taken to document the process and also observe the relationship between the vessels and the surrounding tissue structures. During the process of tissue maceration the casts became increasingly delicate requiring

extreme care in moving the casts. After six weeks (sheep and calf heads) to twenty weeks (adult cow head) the specimens were thoroughly rinsed in tap water to remove debris and chemical residues.

2.2.2.6 Assessment and analysis

The vessels demonstrated were then described, drawn and measured to obtain the internal diameter of the veins. Photographs were taken of the finished casts.

2.2.2.7 Precautions

The injection of the casts with resin was performed in a well-ventilated room and protective clothing including masks, overalls and gloves were worn when handling dangerous chemicals. The solutions of macerated tissue were bagged and sent for incineration.

### 2.2.3 Anatomical study by dissection

2.2.3.1 Principles

Dissection of formalin preserved specimens allows detailed investigation of anatomical structures using specialist instruments and techniques.

2.2.3.2 Specimens

A fresh adult sheep cadaver was obtained from the Langford site abattoir (University of Bristol, UK) following the use of electrical stunning and slaughter by exsanguination. The head and neck were separated from the carcass at the level of the fourth cervical vertebrae.

### 2.2.3.3 Preservation

The specimen was submerged in a bath of twenty-percent formalin and left for one week. It was then thoroughly rinsed in running water before dissection was attempted.

### 2.2.3.4 Dissection

Removal of overlying soft tissues was accomplished using scalpels, dissecting scissors and forceps. Bony structures were removed by using bone rongeurs.

### 2.2.3.5 Precautions

Dissection was performed in a well-ventilated room and protective clothing was worn.

## 2.3 RADIOGRAPHIC STUDY OF VENOUS CEREBRAL DRAINAGE

### 2.3.1 Aims

- To investigate the effects of animal orientation at stunning and slaughter upon uptake and transport of brain tissue emboli by the venous circulation after application of captive bolt gun stunning in sheep.

### 2.3.2 Principles

Administration of a radiographic contrast media into the venous circulation of anaesthetised animals allows the demonstration of cerebral venous outflow by way of radiography.

### 2.3.3 Anaesthesia

The animals were anaesthetised with an intra-muscular induction mixture (ketamine 12mg/ml, xylazine 0.4mg/ml). The animals were then entubated and maintained on a mixture of 1-2% halothane, with 2-4 litres of oxygen and nitrous oxide per minute using a closed breathing system.

### 2.3.4 Animal positioning

Radiographs were taken of anaesthetised sheep in a prone position on the sternum, and after they were transferred to a specially constructed brace that allowed elevation of the animals into various degrees of a "head down" perpendicular position without the necessity for shackling. In addition the brace allowed correct positioning of the radiographic plate under the animals head.

### 2.3.5 Contrast media

A low osmolar, non-ionic, iodinated contrast agent administered by intravenous or intra-cerebral injection was used to demonstrate the venous sinuses and veins draining the head (Omnipaque (iohexol), Nycomed UK Ltd).

#### 2.3.5.1 Administration of contrast media

##### *2.3.5.1.1 Angularis oculi vein injection*

A small incision was made over the vein to allow placement of a 1-inch 22g angiocatheter (Otiva, Johnson and Johnson) in the vein. The catheter was secured in place by means of tape and a small quantity of superglue. A bolus of 10-ml of contrast media was given into each vein. A tape ligature was briefly placed around the animal's neck during injection of contrast media with animal in both positions.

##### *2.3.5.1.2 Dorsal sagittal sinus injection*

A 22-gauge catheter was introduced to the sagittal sinus by way of a burr-hole drilled in the midline of the skull. Reflux of blood from the catheter port suggested correct placement of the catheter in the vein lumen. A bolus injection of 10-ml of contrast media was injected.

##### *2.3.5.1.3 Intra-cranial injection*

The skin over-lying the highest point of the head was incised and reflected to expose the under-lying bone. A small hole of approximately 3mm diameter was then drilled through the skull on the midline and on the highest point of the skull by the use of an electric bur. Penetration of the skull was confirmed by observation of cerebrospinal fluid leaking from the hole.

### 2.3.6 Radiography

Two radiographs were taken in rapid succession of both views for each animal. All radiographs were obtained with a focal-film distance of 100-cm. An automatic film processor and drier were used to develop all films.

## 2.4 SEPARATION AND QUANTIFICATION OF BRAIN TISSUE EMBOLI BY FILTRATION

### 2.4.1 Aims

- To investigate the use of a leukocyte filtration system for separation from blood of brain tissue emboli created by the application of captive bolt gun stunning in sheep.
- To quantify the amount of brain tissue emboli released into the venous circulation after captive bolt gun stunning.

### 2.4.2 Principles

Filtration using leukocyte filters may provide a method for removing tissue fragments of similar and larger size than leukocytes from blood samples. In addition, since the entire blood volume collected after stunning can be passed through a filter the method may allow the quantification of the brain tissue emboli present in the venous blood returning from the head. The use of the pre-filter component of the leukocyte filtration system should allow separation of the larger fragments of brain tissue and enable rapid estimation of the weight of captured tissue in the filter.

### 2.4.3 Anaesthesia

All animals were anaesthetised by intramuscular injection of an induction mixture of ketamine (12mg/kg) and xylazine (0.4mg/kg) and were then maintained by intravenous injection of a guaiphenesin anaesthetic mixture (100:6:3 mixture of 10% guaiphenesin, 10% ketamine and 2% xylazine).

### 2.4.4 Animal preparation

Jugular catheters were implanted in both jugular veins using the previously described technique.

### 2.4.5 Stunning position

Sheep were stunned while in lateral recumbency.

### 2.4.6 Captive bolt guns

Seven of the sheep were stunned by a cartridge-activated penetrating captive bolt gun (Temple Cox, Mark V, Accles and Shelvoke) using a 1.25-grain cartridge and the remaining eight animals were stunned by use of a pneumatically-activated penetrating captive bolt gun (Cash Ramrod, Accles and Shelvoke, UK) using 100 pounds per square inch (psi) air pressure to activate the gun.

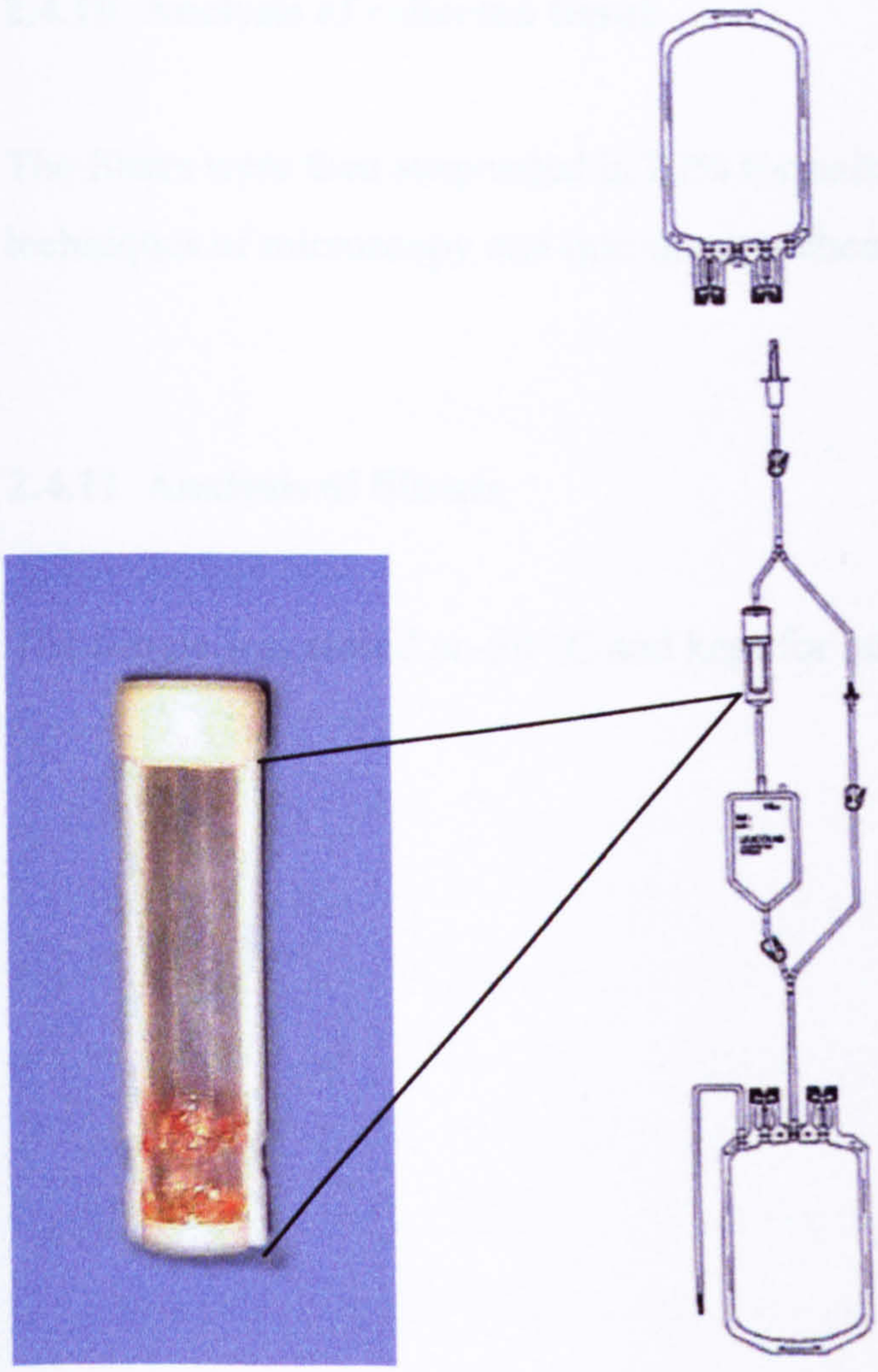
### 2.4.7 Collection of blood

A pre-stun sample blood sample was taken from the jugular cannulas and the animals were immediately stunned by either a pneumatically-activated CBG (Cash ramrod, Accles and Shelvoke, UK) or by a cartridge-activated CBG (Temple Cox, Accles and Shelvoke, UK) and blood collection initiated through the jugular catheters. Sample collection continued over a one minute period into six 250-ml containers in which 25ml of citrate anti-coagulant had been placed.

### 2.4.8 Filters

Standard leukocyte filters (Leucolab (KSV0001XQ, Maco Pharma Laboratories Pharmaceutiques, Tourcoing, France) for use with human blood were used to filter the collected blood samples.

Figure 2.5 Photograph of the pre-filter used to separate the brain tissue emboli (left) and a schematic diagram of the complete filtration system (right) (Leucolab, Macopharma, Tourcoing, France)



2.4.8.1 Filtration

The collected blood was then passed through the pre-filter container which had previously been removed from the outer casing and suspended over an empty collection bottle.

### 2.4.9 Weighing collected tissue

Each filter was weighed and labelled and the weight was recorded. The collected jugular blood was then passed through the filter and the filtrate was collected. The filter

was then flushed with saline to remove any remaining blood cells and then filter was then re-weighed.

### 2.4.10 Analysis of collected tissue

The filters were then suspended in 20% formalin and sent for analysis by the combined techniques of microscopy and immunocytochemistry.

### 2.4.11 Analysis of filtrate

The filtrate was stored at -30° C and kept for analysis by ELISA.

## 2.5 POLYMERASE CHAIN REACTION FOR DETECTION OF BRAIN TISSUE CONTAMINATION

### 2.5.1 Aims

- To validate a real-time PCR assay previously developed for use with bovine DNA using ovine DNA.
- To use the assay to investigate contamination of the ovine carcass with brain tissue emboli following the application of captive bolt gun stunning.

### 2.5.2 Principles

The polymerase chain reaction (PCR) allows amplification of the number of copies of a specific region of DNA (deoxyribonucleic acid), in order to produce enough DNA to be adequately tested. A gene is composed of a precise sequence of four different deoxyribonucleotides within a stretch of DNA. The nucleotides are distinguished by the presence of one of four different nucleotide bases namely Adenine, Thymidine, Cytosine and Guanine. The first step necessary for the PCR is to synthesize "primers" of about 20 nucleotides long using each of the 4 bases, which replicate lengths of DNA on either side of the length to be replicated.

The actual amplification process involves three temperature changes that are repeated over 30-40 cycles to produce a large number of copies of the DNA strand. The first stage involves denaturation of the DNA at 94°C in which the double helix un-winds, this is followed by an annealing step at 54°C in which the primers attach to the DNA template on both sides of the open helix. The polymerase enzyme attaches at this point and begins to replicate the template. The temperature is then raised to 72°C to allow optimal extension of the template from the 5' to 3' direction by the polymerase enzyme. The detection of product DNA may be done using a method of gel electrophoresis or alternatively may use fluorescently labelled probes that attach to the newly synthesised DNA and allow detection and quantification of the product.

### 2.5.3 Samples

Blood and tissues were obtained from the Langford site abattoir. Dr. C.R. Helps, (Department of Clinical Veterinary Science, University of Bristol, UK) donated samples of bovine brain material for use in initial trials of the assay

### 2.5.4 Reagents

- Platinum Q PCR Supermix (Invitrogen, Groningen, The Netherlands)
- Bovine Y primer (Cruachem Ltd, Glasgow, Scotland)
- 28s rDNA primer (Cruachem Ltd,)
- 28s rDNA FQ probe (Cruachem Ltd,)
- SRY FQ probe (Cruachem Ltd,)
- Platinum *Taq* (Invitrogen)
- DNA template
- Water
- $MgCl_2$

### 2.5.5 Extraction

DNA was extracted from tissue samples using QIAamp DNA minikit (Qiagen, Crawley, UK). The protocol described in the Qiagen Dneasy tissue kit handbook for DNA extraction from animal tissues was followed except that only a single elution into 100µl of buffer AE was done.

### 2.5.6 Primers and Probes

Primers to amplify bovine Y-chromosome and bovine 28S DNA sequences were previously designed for investigation of CNS contamination during carcass splitting (Helps et al., 2004). Primers and probes were designed from Genbank sequences (S71489 and AF 154866) using the Primer Express Software version 1.0 (Applied Biosystems, UK).

Table 2.2 Base sequences of primers and probes designed for the real-time PCR assay

| **Primer/Probe** | **Primer/Probe 5' to 3' sequence** |
|---|---|
| Bovine 28S rDNA forward | GGCGAAAGACTAATCGAACCAT |
| Bovine 28S rDNA reverse | CGAGAGCGCCAGCTATCCT |
| Bovine 28S rDNA FQ probe | **Tex Red-** TAGTAGCTGGTTCCCTCCGAAGTTTC CCT-BHQ2 |
| Bovine SRY forward | GTGAACGAAGACGAAAGGTGG |
| Bovine SRY reverse | AGAATGGGCGCTTTTCAGC |
| Bovine SRY FQ probe | **Fam-** TCAGCAAGCAGCTGGGATATGAGTG GAA-BHQ1 |

### 2.5.7 Real-time PCR

An iCylcer IQ system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) was used to amplify the product DNA. The reaction mixture followed that of a previous experiment (Helps et al., 2004). This included 12.5µl of Platinum Q PCR Supermix-UDG, 200nM bovine SRY primers, 200nM bovine 28S rDNA primers, 100nM SRY FQ probe, 200nM 28S rDNA FQ probe, 1.25 units Platinum *Taq*, 3mM $MgCl_2$, 5µl DNA and water to 25µl. The mixture was first incubated at 95°C to activate the Platinum *Taq* then 45 cycles at 95°C for 10 seconds and 60°Cfor 30 cycles. Fluorescence was detected at 530nm and at 630nm at each annealing step. Threshold cycles were calculated using Bio-Rad software.

### 2.5.8 Efficiency of amplification

Ten-fold dilutions of DNA extracted from male sheep brain were prepared in AE buffer to yield dilutions ranging from $10^{-1}$ to $10^{-6}$. These samples including the undiluted male sheep DNA and a negative control were assayed by real-time PCR to produce a standard curve using the Bio-Rad software. A graph of threshold cycle ($C_t$) vs. $\log_{10}$ copy

number of the sample dilution series was produced. The slope of this graph gave the reaction efficiency.

The detection limit of the PCR was calculated using the starting quantity of brain tissue from which the male DNA was extracted and the dilution factor of the final sample in which the SRY DNA was amplified.

## 2.6 INVESTIGATION OF PROTHROMBIN TIME ESTIMATION OF PLASMA AS AN INDIRECT TEST FOR BRAIN TISSUE EMBOLI IN BLOOD

### 2.6.1 Aims

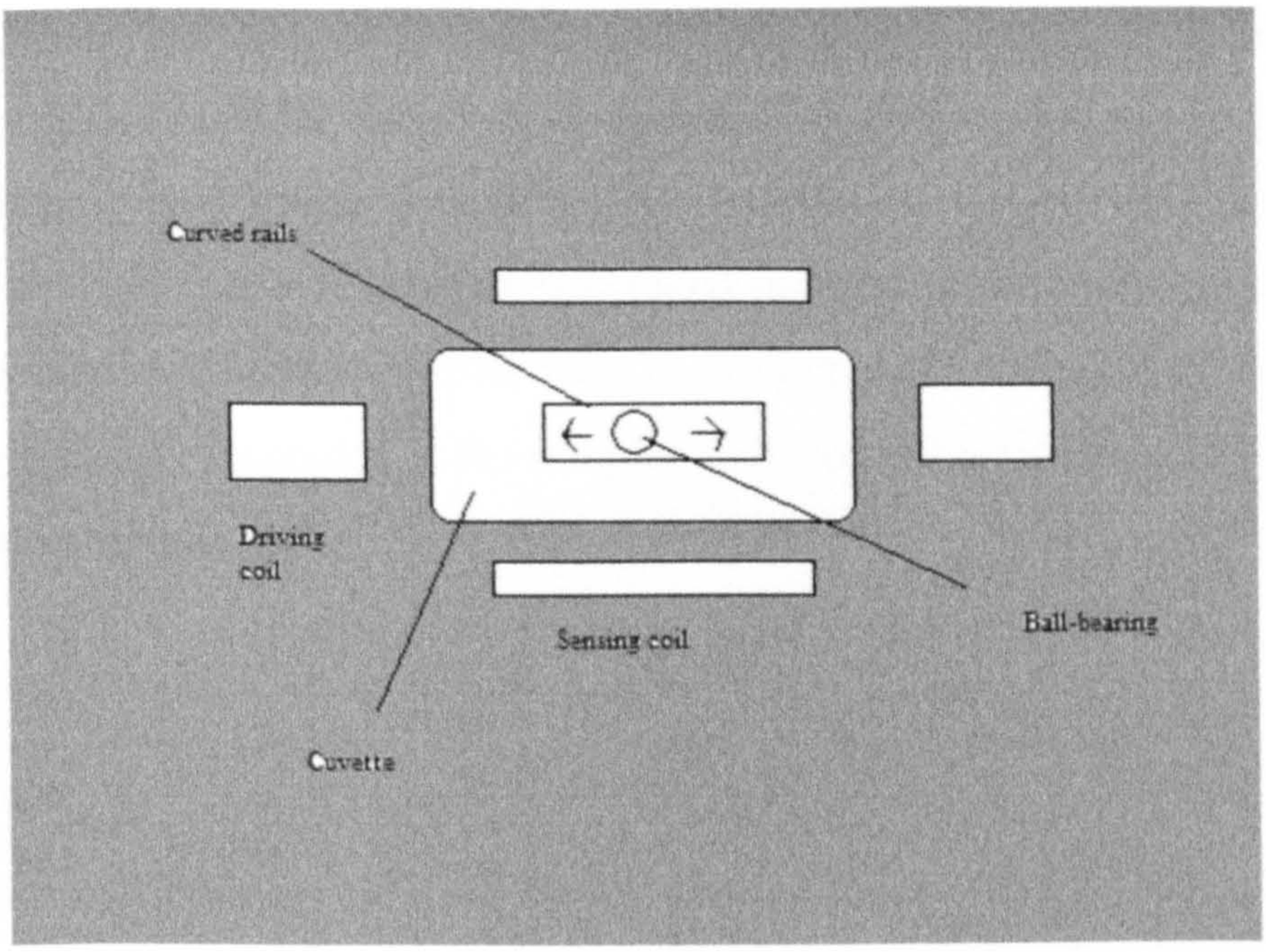
- To investigate the use of prothrombin time estimation as a tool for identifying samples from sheep and cattle in which embolism of brain tissue into the blood occurred following stunning by captive bolt gun.

### 2.6.2 Principles

Screening tests for coagulation involve in-vitro activation of the clotting cascade and measurement of the time until a clot is formed. Prothrombin time (Pt time) is used to evaluate the extrinsic clotting pathway and measures the activity of the coagulation factors, Π, V, VII and X. The addition of calcium thromboplastin to citrated plasma starts the cascade of reactions that ends in the formation of a fibrin clot.

The sample plasma is placed in a well along with a ball bearing that sits on two curved rail tracks. An electro-magnetic field generated by two independent coils is applied alternately to each end of the well to produce a constant swinging pendulum movement of the ball. The oscillation of the ball is recorded through an inductive displacement sensor (Figure 2.6). Clotting measurement is based upon the decreased amplitude of the ball oscillations as the viscosity of the sample increases with clotting. An algorithm is used to calculate the clotting time.

Figure 2.6 Diagram indicating the determination of clotting measurement by recording ball oscillations through an inductive displacement sensor (ST art 4, Diagnostica Stago, Asnières, France)




To minimise instrumental and reagent differences in the evaluation of prothrombin time the World Health Organisation has introduced a method of standardisation known as the International Normalised Ratio (INR) to express PT time when human plasma samples are analysed.

In this study PT times are not standardised since comparison of the results with other assay systems was not required and a similar method of standardisation does not exist for animal plasma samples.

### 2.6.3 Reagents

- Rabbit brain thromboplastin (Neoplastine CI plus, Diagnostica Stago, Asineres, France)
- Owens Veronal buffer (Diagnostica Stago)
- STA Unicalibrator (Diagnostica Stago)
- Distilled water

### 2.6.4 Samples

Cattle and sheep plasma was obtained from animals during the sampling for brain tissue embolism after CBG stunning in which blood samples were collected into citrated collection bottles. The samples for the coagulopathy study were chosen from the stored plasma samples and included samples that were previously found to have been taken from animals positive for brain tissue embolism by either or by both of the analytic methods used. A selection of samples designated as negative for brain tissue embolism were also included. All seven samples from each sample animal including the pre-stunning samples were included in the samples analysed except in cases in which plasma samples were missing.

#### 2.6.4.1 Storage

Plasma was rapid frozen in liquid nitrogen before being transferred to freezers at -30°C. Samples were stored for up to 18 months before assay for PT time.

### 2.6.5 Controls

The STA Unicalibrator supplied with the kit was assayed in duplicate as the positive control sample and duplicate samples of distilled water provided negative control samples before each use of the machine to check the accuracy and reproducibility of results.

### 2.6.6 Clotting time determination

Plasma samples were thawed at room temperature and then centrifuged at *800x g* before use in the assay. Samples were run in duplicate on a coagulation analyser (ST art 4, Diagnostica Stago, Asnières, France).

The activating reagent was first warmed to thirty seven degrees and was mixed by magnetic stirrer after which 50-µl of sample plasma in citrate was then added in duplicate to the cuvettes in which a ball bearing had previously been placed. The

cuvette was then warmed for a period of at least fifty seconds to reach a temperature of 37°C. After the required temperature was reached, the cuvette was moved to the test area and 100-µl of Neoplastin Plus reagent was added. Simultaneously the magnetic field was activated causing oscillation of a ball bearing previously placed in the well. The movement of this ball bearing on curved rail tracks on the base of the cuvette well was monitored by an inductive displacement sensor built into the machine. As the viscosity of the sample increases as the clotting cascade is activated the ball bearings rate of oscillation is slowed and the PT time calculated and recorded.

### 2.6.7 Performance

Within assay and inter-assay coefficients of variation were calculated by analysis of 20 aliquots of the unicalibrator on the same day and on three separate days respectively.

### 2.6.8 Statistical analysis

General linear models as well as paired t-tests were used to analyse the data (Minitab Release 14).

# CHAPTER 3 EXPERIMENTAL

## 3.1 DETECTION OF BRAIN TISUE CONTAMINATION IN BLOOD AND OTHER TISSUES USING AN ELISA AND A COMBINATION OF MICROSCOPY AND IMMUNOCYTOCHEMISTRY

### 3.1.1 Introduction

In this study the potential for brain tissue fragments to be disseminated in the carcasses of cattle and sheep after use of stunning methods currently in use or that are authorised for use in the UK was examined.

Brain tissue contamination was detected in blood and other tissues using an ELISA developed for detection of the CNS specific protein glial fibrillary acidic protein (GFAP). In addition, a proportion of samples from each animal was also analysed for the presence of brain tissue using a combined technique of microscopy and immunocytochemistry. In this technique, the central nervous system (CNS) tissue specific proteins S-100β and neurofilament protein were used as antigen markers for the presence of brain tissue.

As part of validation exercises of both analytic methods used in this group of experiments, a series of dilutions of macerated brain tissue in blood was prepared and subsequently analysed by ELISA and also by the technique of microscopy and immunocytochemistry.

### 3.1.2 Aims

- To determine the frequency of brain tissue embolism after captive bolt gun stunning in cattle and sheep.
- To investigate the potential for brain tissue fragments to penetrate the lungs and enter the aortic circulation.
- To investigate the potential of electrical stunning to cause entry of brain tissue fragments into the venous circulation.
- To investigate the potential for organ contamination with brain tissue fragments.

### 3.1.3 Methodology

#### 3.1.3.1 Jugular vein blood sampling following stunning

For details of the method refer to 2.1

##### 3.1.3.1.1 *Captive bolt gun stunning*

*3.1.3.1.1.1 Cattle*

Jugular venous samples were taken from two hundred cattle of which one hundred were stunned with a penetrating captive bolt gun and the remaining one hundred by a non-penetrating captive bolt gun. All samples were taken from cattle at an over thirty months scheme (OTMS) designated abattoir (Jarrett's abattoir, Keynsham, Bristol, UK) approved for this purpose by the UK Home Office. Between six and twelve cattle were sampled on each visit and included a variety of breeds and a range of ages above thirty months. All animals sampled were female and the majority were of the Holstein-Friesian breed.

*3.1.3.1.1.2 Sheep*

Jugular venous samples were taken from two hundred adult sheep, one hundred after stunning with a cartridge-activated penetrating CBG and one hundred after the use of a pneumatically-activated penetrating CBG without air-injection facility. All sheep used in the experiment were animals previously selected for culling due to a variety of causes including barrenness, 'poor doers' and disease. A variety of common breeds were obtained from a farm close to the Langford site (University of Bristol, UK). No attempts were made to restrict the age of animals included in the study although a size restriction of between twenty and sixty kilograms was imposed. The weight and gender of animals was noted. Sampling of sheep was conducted from February 2002 to March 2003. Between six and fifteen animals were sampled on any given occasion.

#### 3.1.3.1.2 *Electrical stunning*

At a commercial abattoir (St. Merryn Meat Ltd, Merthyr Tydfil, Wales)in which a fully automated electrical stunning device (Jarvis, New Zealand) is used, twenty cattle heads were removed from the line immediately following slaughter, exsanguination and subsequent decapitation and then examined for brain tissue and vessel trauma.
At the same abattoir on a separate occasion approximately five hundred millilitres of exsanguinated blood was collected as rapidly as possible following electrical stunning and sticking from twenty animals randomly selected from the line. Practical restrictions at the abattoir prevented a pre-stun sample from being taken.

### 3.1.3.2 Aortic blood sampling following injection of brain suspension and simultaneous stunning

For details of the method refer to 2.1

#### *3.1.3.2.1 Cattle*

Prepared suspensions of brain tissue were injected into the jugular vein blood of ten cattle that were then each stunned by a penetrating captive bolt gun. Sampling of aortic blood began immediately and continued for two minutes.

#### *3.1.3.2.2 Sheep*

##### *3.1.3.2.2.1 Jugular vein injection*

Prepared suspensions of brain tissue were injected into the jugular vein blood of eleven adult sheep that were then each stunned by a penetrating captive bolt gun. Sampling of aortic blood began immediately and continued for the full duration of blood flow.

*3.1.3.2.2.2 Intra-cranial injection with occluded jugular drainage*

Three adult sheep of 30 to 40-kg were used in this study. Prepared suspensions of brain tissue were injected into the bolt-hole of a single sheep and into the angularis oculi veins of two further sheep following stunning by a penetrating captive bolt gun. Both jugular veins of all sheep had previously been exposed and tied off in order for the vertebral venous system to act as the sole drainage portal from the brain. Sampling of aortic blood began immediately and continued for the full duration of blood flow.

**3.1.3.3 Organ sampling following injection of brain tissue suspension and stunning**

Five adult sheep were used in this study in which brain tissue suspensions were injected into the cerebral sinuses by way of the angularis oculi veins. The animals were then stunned and slaughtered by exsanguination. Samples of exsanguinated blood were taken and at post-mortem samples of cervical lymph nodes, spleen, liver and kidney were taken from each carcass.

**3.1.3.4 Validation**

As part of validation exercises performed on both analytic methods used in this group of experiments a series of dilutions of macerated brain tissue in blood was prepared and analysed by ELISA and also by the technique of microscopy and immunocytochemistry. In addition, brain tissue suspensions of known concentration were injected into lymph node specimens previously harvested from bovine carcasses that were then also analysed by ELISA.

### 3.1.4 Results

#### 3.1.4.1 Jugular-vein blood sampling of cattle following captive bolt gun stunning

##### *3.1.4.1.1 Penetrating CBG group*

###### *3.1.4.1.1.1 ELISA*

Two animals (2/100) were found to have raised levels of GFAP of which one animal (65) demonstrated increased levels of GFAP from twenty seconds to forty seconds of sampling time with a peak during twenty to thirty seconds of sampling time (Fig 3.1). The second positive animal (67) demonstrated increased GFAP levels in the sample taken at twenty to thirty seconds after stunning.

Figure 3.1 Graph demonstrating the concentrations of GFAP detected by ELISA in samples taken after captive bolt gun stunning from a single cow (65)

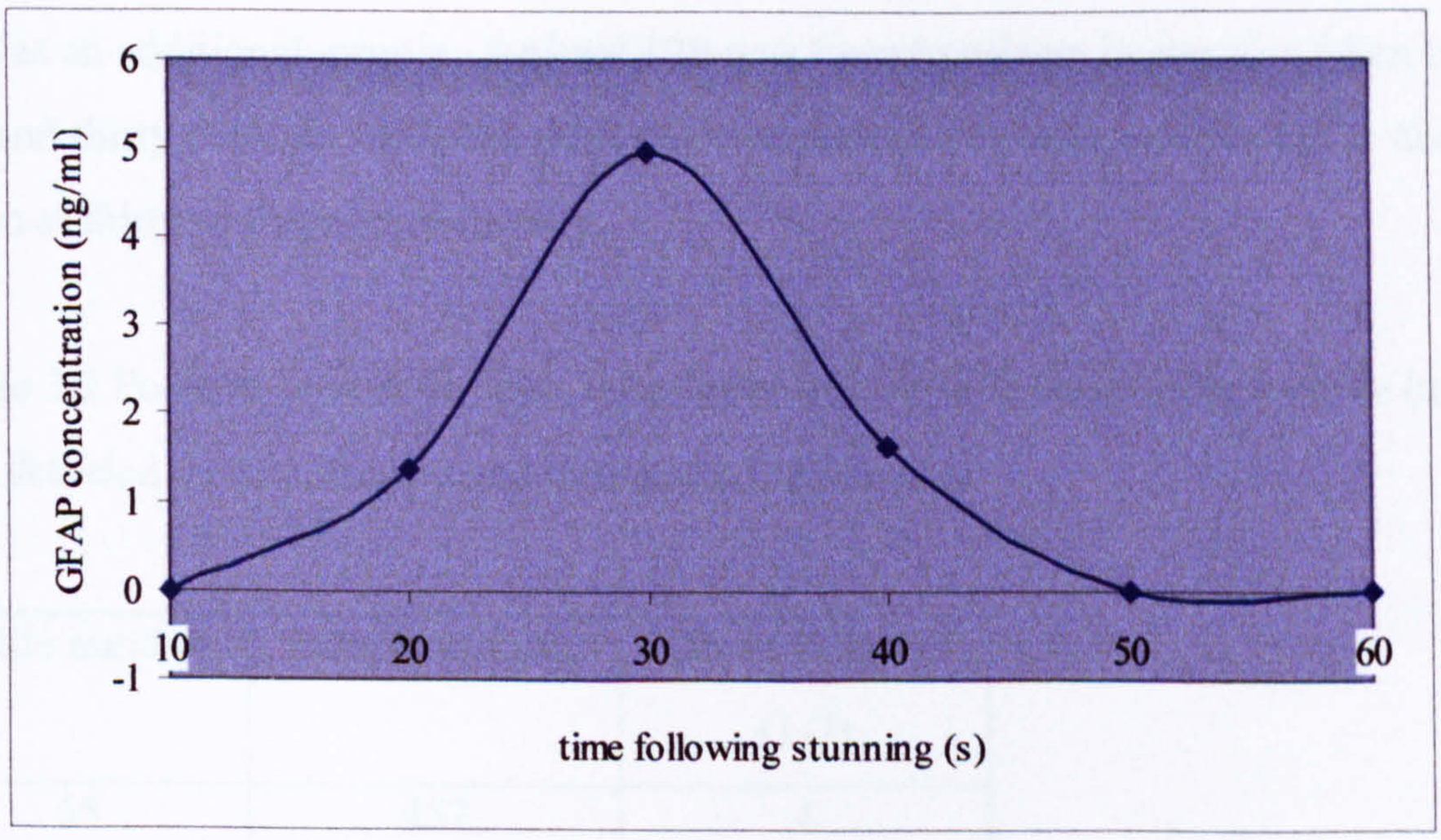


Table 3.1 Positive bovine samples taken after the use of a penetrating captive bolt gun and detected by ELISA

| Cattle number | Sample number | Positive by GFAP ELISA (ng/ml) | Timed aliquot *(1-7) |
|---|---|---|---|
| 65 | 451 | 1.37 | 3 |
| | 452 | 4.98 | 4 |
| | 453 | 1.63 | 5 |
| 67 | 466 | 2.13 | 4 |

*Aliquot 1= pre-stun sample

*3.1.4.1.1.2 Microscopy and immunocytochemistry*

Three (3/100) animals were found positive by the complementary methods of microscopy and immunocytochemistry of which one was also positive by ELISA (cow number 65). Animal 65 produced positive samples in aliquots taken at twenty to forty seconds and also in the final sample taken at fifty to sixty seconds. This final sample was observed to be clotted at the time of processing of samples and consequently was sent as an additional sample. Animal 129 was found positive in samples taken between ten and thirty seconds while the third positive animal 133 was positive in the aliquot taken at thirty to forty seconds only.

Table 3.2 Positive bovine samples taken after the use of a penetrating captive bolt gun and detected by microscopy and immunocytochemistry

| Cattle number | Sample number | Timed aliquot* (1-7) |
|---|---|---|
| 65 | 452 | 4 |
| | 453 | 5 |
| | 455 | 7 |
| 129 | 899 | 3 |
| | 900 | 4 |
| 133 | 929 | 5 |

*Aliquot 1= pre-stun sample

#### *3.1.4.1.2 Non-penetrating CBG group*

##### *3.1.4.1.2.1 ELISA*

No animals (0/100) were found to be positive by ELISA of blood samples.
All samples analysed by GFAP ELISA were found to have levels of GFAP consistent with background concentrations.

##### *3.1.4.1.2.2 Microscopy and immunocytochemistry*

Two (2/100) animals were found positive by the complementary methods of microscopy and immunocytochemistry. Brain tissue fragments were observed in Cytoblock sections prepared from blood samples obtained from animals 88 and 137 respectively. A single sample taken between thirty to forty seconds of sampling time was found to contain fragments of meninges from animal 88 while a single aliquot taken between ten and twenty seconds of sampling time was found to be positive for brain tissue fragments from animal 137.

Table 3.3 Positive bovine samples taken after the use of a non-penetrating captive bolt gun and detected by microscopy and immunocytochemistry

| Cattle number | Sample number | Timed aliquot* |
|---|---|---|
| 88 | 614 (identified as meninges by microscopy) | 5 |
| 137 | 955 | 3 |

*Aliquot 1 = pre-stun sample

#### *3.1.4.1.3 Combined results*

Four (4/100) animals were identified as having embolism of brain tissue fragments after the use of the penetrating CBG [Frequency of 0.04, 95% confidence interval of 0.016 to 0.098].

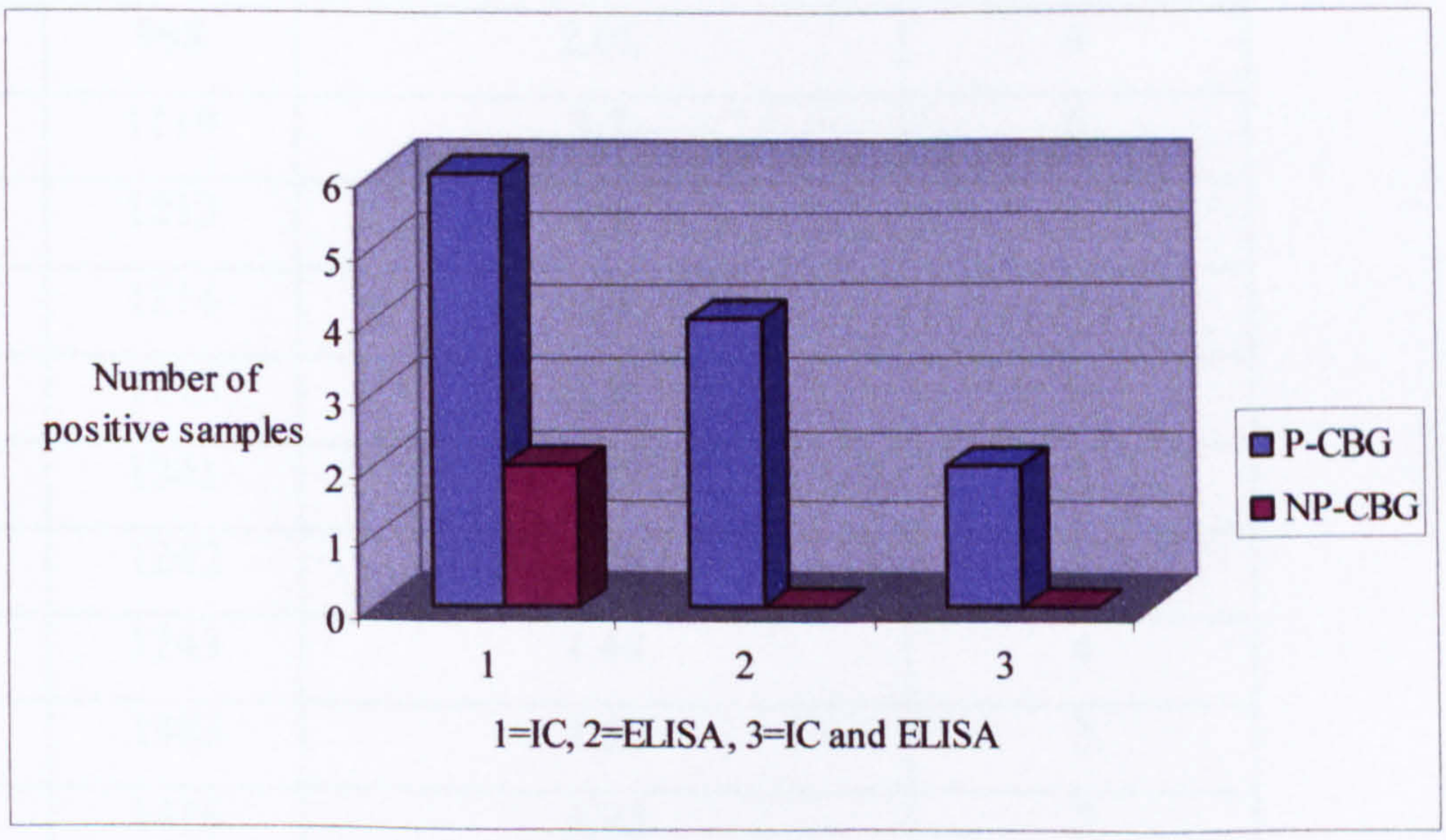
Two (2/100) animals were identified as having embolism of brain tissue fragments after the use of the non-penetrating CBG [Frequency of 0.02, 95% confidence interval of 0.006-0.07].

There was found to be no significant difference between the frequencies of brain tissue embolism observed with use of each captive bolt gun (Fishers exact test, p= 0.683).

Table 3.4 Calculations of power based on actual and larger cattle sample sizes

| Proportion | Sample size | Power |
|---|---|---|
| 0.02 | 100 | 0.131236 |
| 0.02 | 500 | 0.457704 |
| 0.02 | 1000 | 0.746269 |
| 0.02 | 2000 | 0.959990 |

Figure 3.2 Individual samples in which brain tissue emboli were detected by ELISA (2), microscopy and immunocytochemistry (1) or by both analytic methods (3) following use of the penetrating (P) or non-penetrating captive bolt gun (NP-CBG)



### 3.1.4.2 Jugular-vein blood sampling of sheep following captive bolt gun stunning

#### *3.1.4.2.1 Pneumatically-activated CBG group*

##### *3.1.4.2.1.1 ELISA*

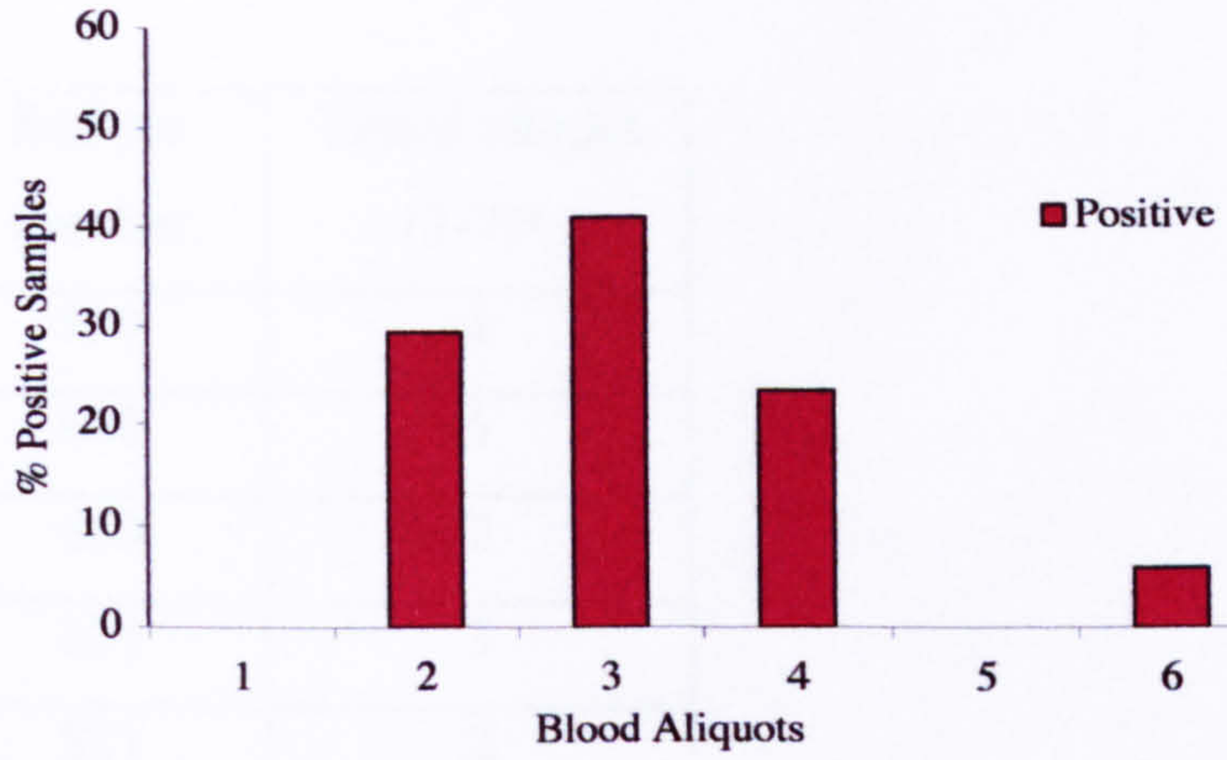
Seventeen samples from ten (10/100) different animals demonstrated significantly elevated levels of GFAP above background levels.

Table 3.5 Positive ovine samples taken after the use of a pneumatically-activated penetrating captive bolt gun and detected by ELISA

| Sheep number | Sample number | Positive by GFAP ELISA(ng/ml) | Timed aliquot (1-7)* |
|---|---|---|---|
| 86 | 599 | 1.9 | 4 |
| 118 | 821 | 50.6 | 2 |
| | 822 | 5.11 | 3 |
| 121 | 842 | 3.21 | 2 |
| 137 | 955 | 1.21 | 3 |
| 143 | 997 | 14 | 3 |
| | 998 | 2.01 | 4 |
| 160 | 1119 | 3.2 | 6 |
| 174 | 1213 | 4.5 | 2 |
| | 1214 | 9.3 | 3 |
| | 1215 | 2.9 | 4 |
| 178 | 1241 | 5.47 | 2 |
| | 1242 | 1.28 | 3 |
| | 1243 | 1.44 | 4 |
| 198 | 1383 | 1.52 | 3 |
| 203 | 1416 | 1.93 | 2 |
| | 1417 | 2.42 | 3 |

* Aliquot 1= pre-stun sample

Figure 3.3 Percentage frequencies of positive samples in each timed aliquot identified by ELISA after pneumatically-activated CBG stunning




*3.1.4.2.1.2 Microscopy and immunocytochemistry*

Twenty-one samples from thirteen (13/100) different animals were found to contain visible fragments of brain tissue.

Table 3.6 Positive ovine samples taken after use of a pneumatically-activated penetrating captive bolt gun and detected by microscopy and immunocytochemistry

| Sheep number | Sample number | Timed aliquot (1-7)* |
|---|---|---|
| 86 | 599 | 4 |
| | 600 | 5 |
| 115 | 800 | 2 |
| | 801 | 3 |
| 118 | 821 | 2 |
| 121 | 842 | 2 |
| | 843 | 3 |
| 129 | 900 | 4 |
| 137 | 954 | 2 |
| 143 | 997 | 3 |
| | 998 | 4 |
| | 999 | 5 |
| 163 | 1137 | 3 |
| 174 | 1214 | 3 |
| 178 | 1241 | 2 |
| | 1242 | 3 |
| 184 | 1283 | 2 |
| 198 | 1383 | 3 |
| 203 | 1416 | 2 |
| | 1417 | 3 |
| | 1418 | 4 |

* Aliquot 1= pre-stun sample

#### *3.1.4.2.2 Cartridge-activated CBG group*

##### *3.1.4.2.2.1 ELISA*

Twenty-five samples from fourteen (14/100) different animals demonstrated significantly elevated levels of GFAP above background levels.

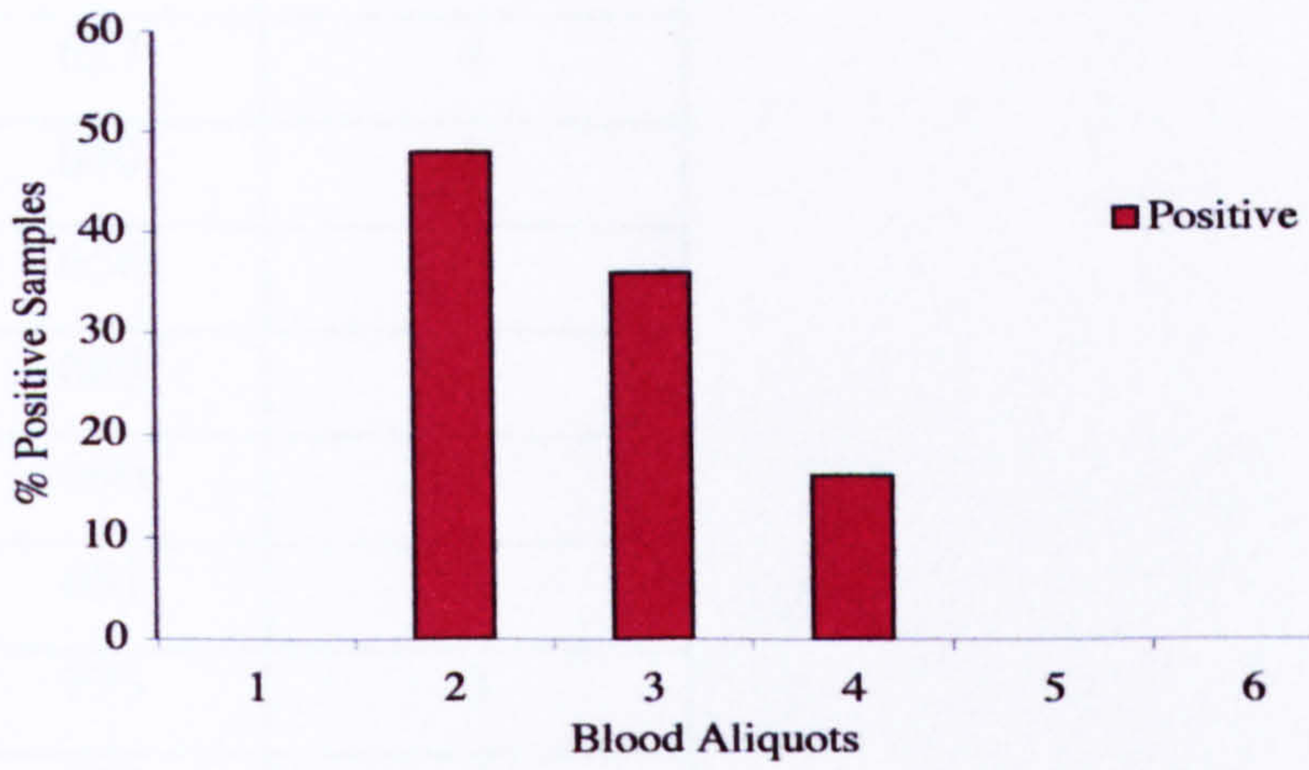
Table 3.7 Positive ovine samples taken after use of a cartridge-activated penetrating captive bolt gun and detected by ELISA

| Sheep number | Sample number | Positive by GFAP ELISA (ng/ml) | Timed aliquot (1-7)* |
|---|---|---|---|
| 71 | 492 | 24.32 | 2 |
| 74 | 513 | 1.78 | 2 |
| | 514 | 1.124 | 2 |
| 75 | 520 | 9.82 | 2 |
| | 521 | 2.19 | 3 |
| 80 | 555 | 18.84 | 2 |
| | 556 | 9.74 | 3 |
| | 557 | 1.814 | 4 |
| 82 | 569 | 4.66 | 2 |
| | 570 | 23.78 | 3 |
| 83 | 576 | 29.84 | 2 |
| | 577 | 2.175 | 3 |
| 90 | 627 | 2.284 | 4 |
| 92 | 640 | 1.65 | 3 |
| 96 | 667 | 1.17 | 2 |
| | 668 | 1.55 | 3 |
| 99 | 688 | 244.5 | 2 |
| | 689 | 13.2 | 3 |
| 104 | 723 | 3.716 | 2 |
| 111 | 772 | 23.97 | 2 |
| | 773 | 2.96 | 3 |

| | | | |
|---|---|---|---|
| 130 | 905 | 25.4 | 2 |
| | 906 | 12.59 | 3 |
| | 907 | 26.2 | 4 |
| 161 | 1124 | 4.5 | 4 |

* Aliquot 1= pre-stun sample

Figure 3.4 Percentage frequencies of positive samples in each timed aliquot identified by ELISA after cartridge-activated CBG stunning




*3.1.4.2.2.1 Microscopy and immunocytochemistry*

Twenty-eight samples from eighteen (18/100) different animals were found to contain visible fragments of brain tissue.

Table 3.8 Positive samples taken after use of a cartridge-activated penetrating captive bolt gun and detected by microscopy and immunocytochemistry

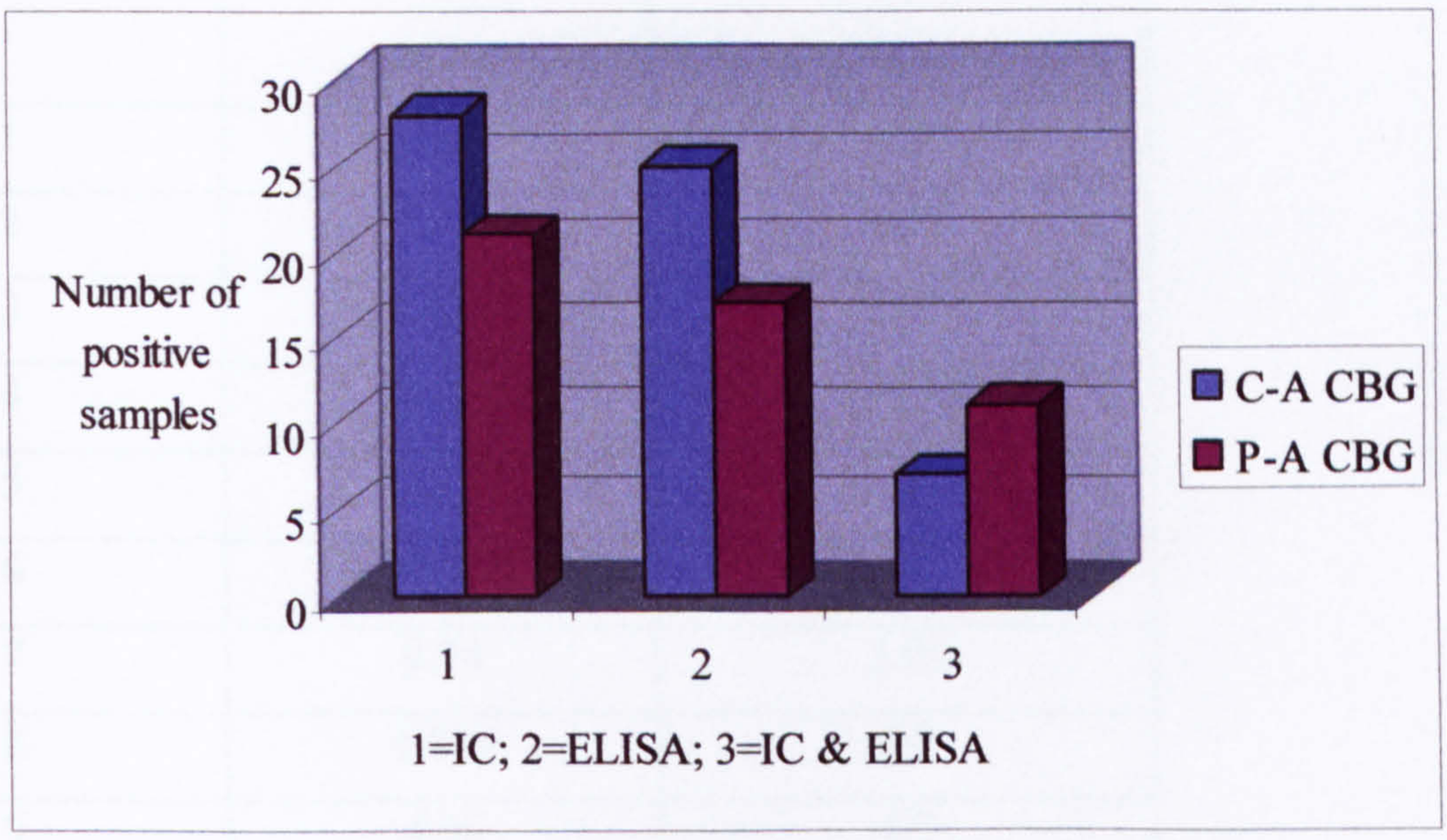
| Sheep number | Sample number | Timed aliquot (1-7)* |
|---|---|---|
| 70 | 487 | 4 |
| | 488 | 5 |
| 75 | 522 | 4 |
| 81 | 564 | 4 |
| 82 | 571 | 4 |
| 83 | 578 | 4 |
| 90 | 627 | 4 |
| 92 | 640 | 3 |
| 94 | 656 | 5 |
| 99 | 689 | 3 |
| | 690 | 4 |
| | 691 | 5 |
| 111 | 773 | 3 |
| | 774 | 4 |
| 114 | 794 | 3 |
| 130 | 905 | 2 |
| | 906 | 3 |
| | 908 | 5 |
| 132 | 919 | 2 |
| | 920 | 3 |
| 155 | 1080 | 2 |
| | 921 | 4 |
| 161 | 1124 | 4 |
| 169 | 1178 | 2 |
| | 1179 | 3 |
| | 1180 | 4 |
| 183 | 1276 | 2 |
| 200 | 1396 | 3 |

* Aliquot 1= pre-stun sample

### *3.1.4.2.3 Combined results*

A total of fourteen (14/100) animals were found positive for brain tissue embolism after the use of the pneumatically-activated CBG [Prevalence of 0.14 with 95% confidence interval of 0.085 to 0.22]. 9 animals (64%) were confirmed positive by both assays.
A total of twenty-three (23/100) animals were found positive for brain tissue embolism after use of the cartridge-activated CBG [Prevalence of 0.23 with 95% confidence interval 0.16to 0.32]. 9 animals (39%) were confirmed positive by both assays.

**Figure 3.5** Individual samples in which brain tissue emboli were detected by microscopy and immunocytochemistry (1), ELISA (2), or by both analytic methods (3) following use of the cartridge-activated (C-A) CBG or pneumatically-activated (P-A) CBG




There was found to be no significant difference between the frequencies of brain tissue embolism observed with use of each captive bolt gun (Fishers exact test, p= 0.099)

Table 3.9 Calculations of power based on actual and larger sheep sample sizes

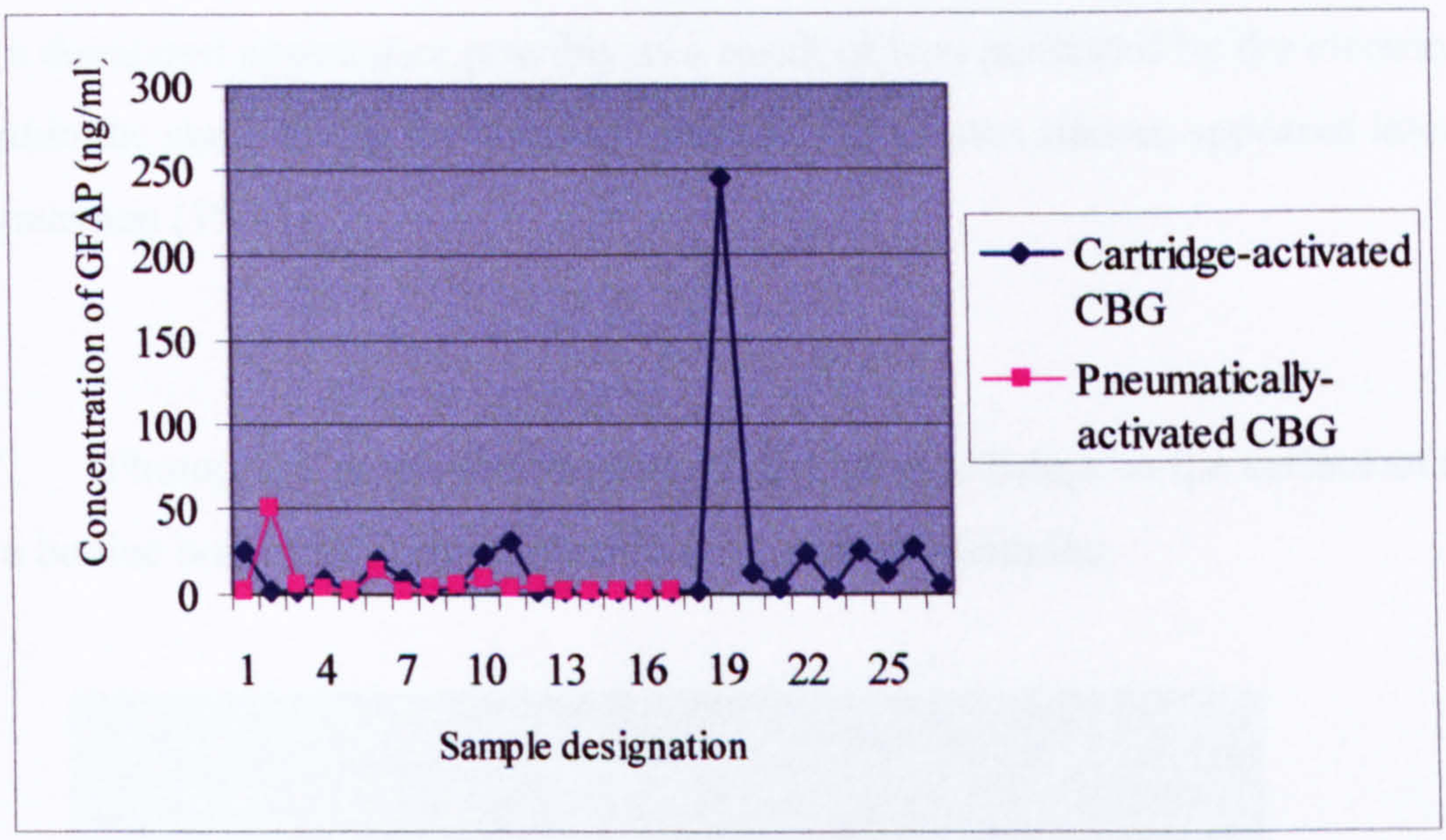
| Proportion | Sample size | Power |
|---|---|---|
| 0.14 | 100 | 0.373416 |
| 0.14 | 500 | 0.956954 |
| 0.14 | 1000 | 0.999412 |

Table 3.10 Concentrations of GFAP detected by ELISA in ovine blood samples taken after stunning by captive bolt gun

| Sample designation | Cartridge-activated captive bolt gun [Concentration of GFAP (ng/ml)] | Pneumatically-activated captive bolt gun [Concentration of GFAP (ng/ml)] |
|---|---|---|
| 1 | 24.32 | 1.9 |
| 2 | 1.78 | 50.6 |
| 3 | 1.124 | 5.11 |
| 4 | 9.82 | 3.21 |
| 5 | 2.19 | 1.21 |
| 6 | 18.84 | 14 |
| 7 | 9.74 | 2.01 |
| 8 | 1.814 | 3.2 |
| 9 | 4.66 | 4.5 |
| 10 | 23.78 | 9.3 |
| 11 | 29.84 | 2.9 |
| 12 | 2.175 | 5.47 |
| 13 | 2.284 | 1.28 |
| 14 | 2.175 | 1.44 |
| 15 | 2.284 | 1.52 |
| 16 | 1.65 | 1.93 |
| 17 | 1.17 | 2.42 |
| 18 | 1.55 | |

| | | |
|---|---|---|
| 19 | 244.5 | |
| 20 | 13.2 | |
| 21 | 3.716 | |
| 22 | 23.97 | |
| 23 | 2.96 | |
| 24 | 25.4 | |
| 25 | 12.59 | |
| 26 | 26.2 | |

Figure 3.6 Concentrations of GFAP detected by ELISA in ovine blood samples after stunning by cartridge-activated or pneumatically-activated captive bolt guns




A Mann-Whitney 2 sample rank test was performed on the GFAP concentrations detected from each CBG group of positive samples. No significant difference was found between the medians (p=0.197).

### 3.1.4.3 Blood sampling of cattle following electrical stunning and sticking

Raised levels of GFAP were detected in a blood sample collected from a single animal (1/20). All other samples had levels of GFAP consistent with the background level.

Table 3.11 Elevated levels of GFAP detected by ELISA in bovine blood sample C (e) 36

| Cattle designation | Sample number | OD value by GFAP ELISA | Positive by GFAP ELISA (ng/ml) |
|---|---|---|---|
| 18 | C(e)36 | 0.63 | 2.5 |

### 3.1.4.4 Examination of cattle brains following electrical stunning and slaughter

Superficial focal sub-arachnoid haemorrhages on the surface of the cortex were observed in eleven (11/15) specimens. Although four (4/15) specimens had no macroscopic evidence of trauma or haemorrhage small blood vessels on the surface of the cortex appeared dilated and congested. In six (6/15) specimens the brain tissue displayed a denatured appearance possibly as a result of heat generated by the electrical current within the skull during the stunning process. All venous sinuses appeared intact in every specimen (15/15).

Figure3.7 Photograph demonstrating superficial haemorrhages on the surface of the cortex of a bovine brain following application of electrical stunning

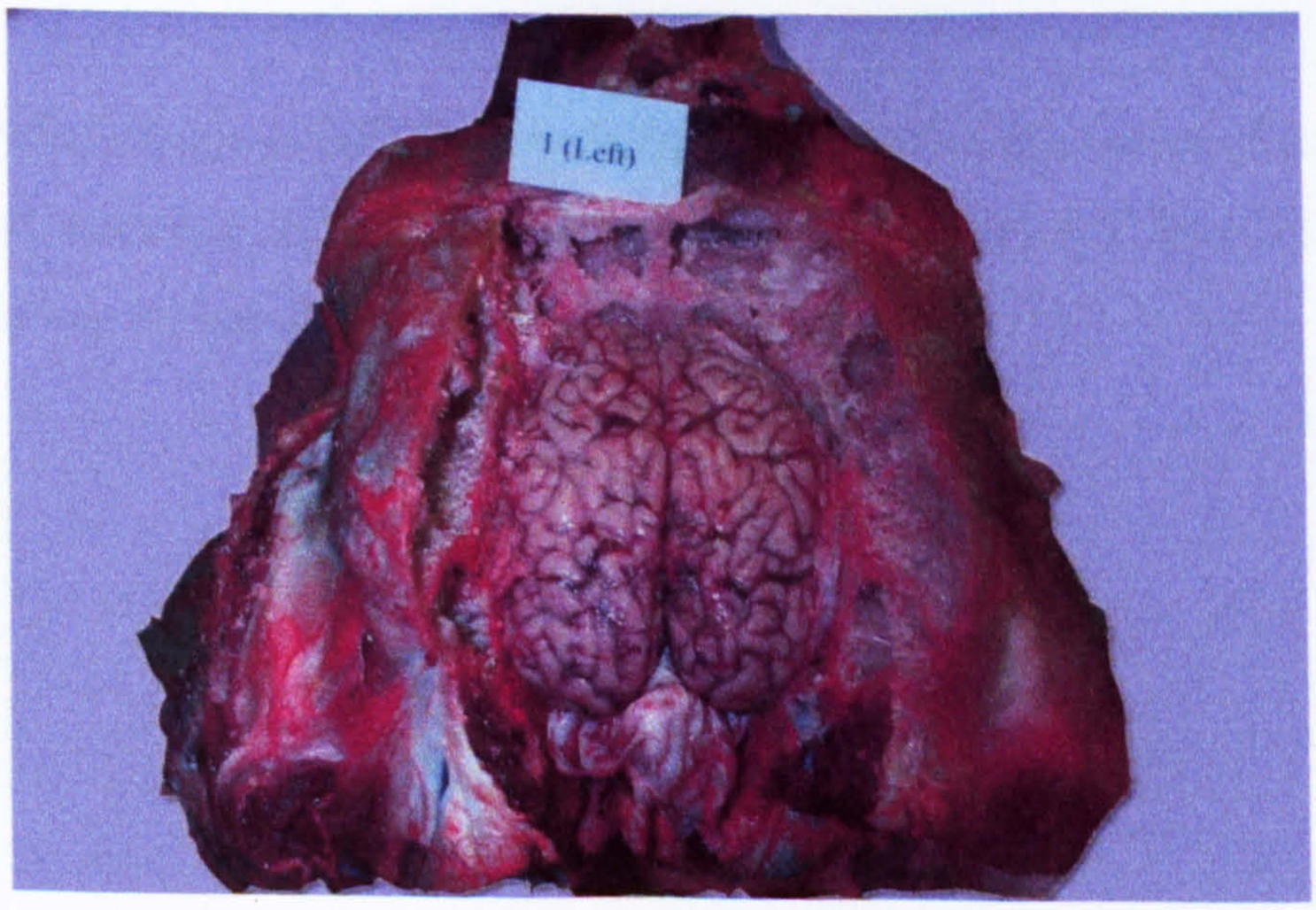
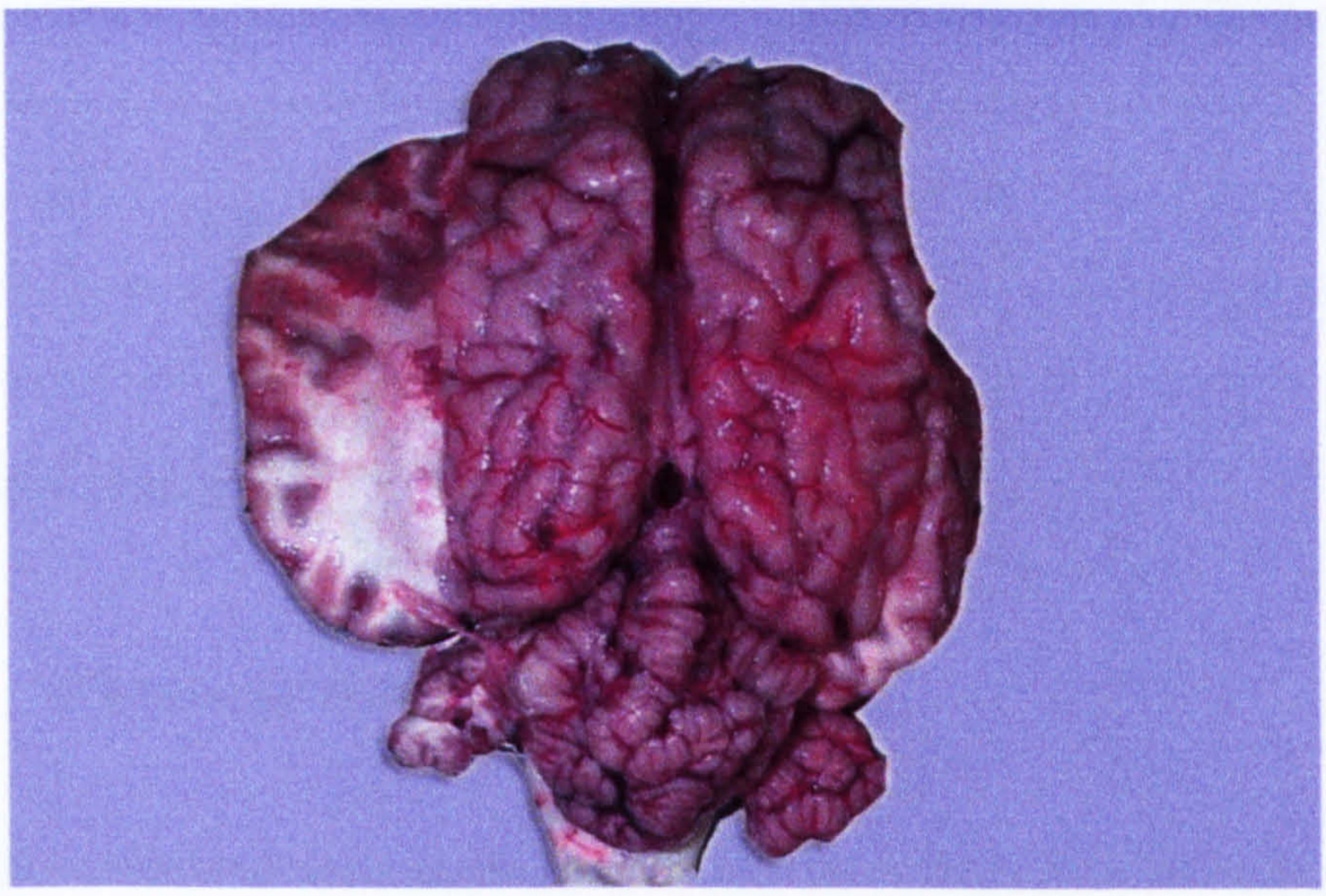


Figure 3.8 Photograph demonstrating congested and dilated appearance of small arterioles on the surface of the cortex of a cattle brain following application of electrical stunning



**3.1.4.5 Aortic blood sampling of cattle following injection of brain suspensions and stunning**

***3.1.4.5.1 ELISA***

Moderately raised levels of GFAP were detected in samples from three of ten (3/10) animals tested, namely (C(c) 30, C (i) 107, C (j) 120, C (j) 121 & C (j) 122). All other samples including all pre-stun samples were negative.

Table 3.12 Positive bovine aortic blood samples detected by ELISA

| Cattle designation | Sample Number | Positive by GFAP ELISA (ng/ml) | Timed aliquot *1-7 |
|---|---|---|---|
| c | 30 | 5 | 3 |
| i | 107 | 1.68 | 2 |
| j | 120 | 2.3 | 2 |
| | 121 | 1.1 | 3 |
| | 122 | 1.48 | 4 |

* Sample 1 refers to the pre-stun sample

#### 3.1.4.5.2 *Microscopy and immunocytochemistry*

All Cytoblocks examined were negative for brain tissue fragments from all ten cattle sampled (0/10).

### 3.1.4.6 Aortic blood sampling of sheep following injection of brain suspensions and stunning

#### 3.1.4.6.1 *ELISA*

Central nervous system protein was detected in blood samples from six of eleven (6/11) animals tested namely, [S (a), S (b), S (d), S (f), S (h) and S (k). In five animals, S (a), S (b), S (d), S (f) and S (h), GFAP was detected within the first minute following injection of brain suspension. The GFAP concentrations of the positive control sample S (g) demonstrate a consistent increase of GFAP concentration with increasing volume of brain suspension.

Table 3.13 Concentration of GFAP (ng/ml) in positive blood samples taken following stunning for the maximum duration of blood flow from each animal and later analysed by ELISA and by a combination of microscopy and immunocytochemistry

| Time in successive minutes of each sample | Sheep designation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | a | b | d | f | h* | k | l* | g |
| 1 | 153.2 | 58.02 | 0.603 | 1.004 | 1.516 | nd | nd | 15.96 |
| 2 | 228.8 | 64.58 | 2.87 | 1.084 | 2.491 | nd | nd | 33.90 |
| 3 | 149.4 | 46.24 | 4.56 | 1.03 | 2.117 | 0.806 | nd | 51.60 |
| 4 | | | 4.76 | 1.033 | 1.573 | | nd | 270.50 |
| 5 | | | 2.87 | 0.84 | 2.06 | | nd | 278.50 |
| 6 | | | 3.16 | 0.93 | | | nd | 285.90 |
| 7 | | | 2.79 | | | | nd | |
| 8 | | | 1.14 | | | | nd | |
| 9 | | | 5.13 | | | | nd | |
| 10 | | | 2.71 | | | | nd | |
| 11 | | | 5.89 | | | | nd | |
| 12 | | | 3.98 | | | | nd | |

nd= Not detectable

* Positive on immunocytochemistry

Figure 3.9 Concentrations of GFAP detected by ELISA in sequential aliquots of aortic blood collected over the total duration of blood flow from three sheep

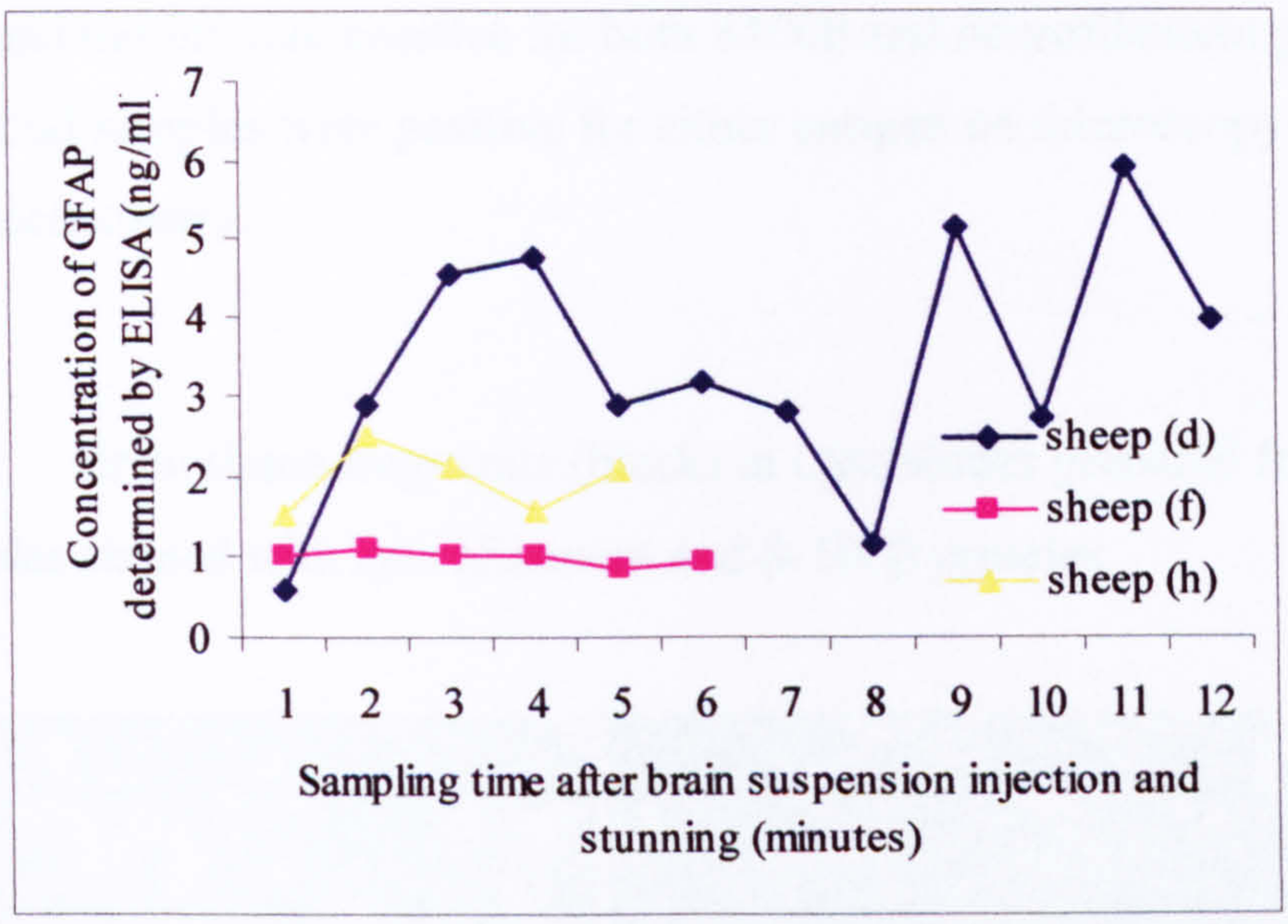
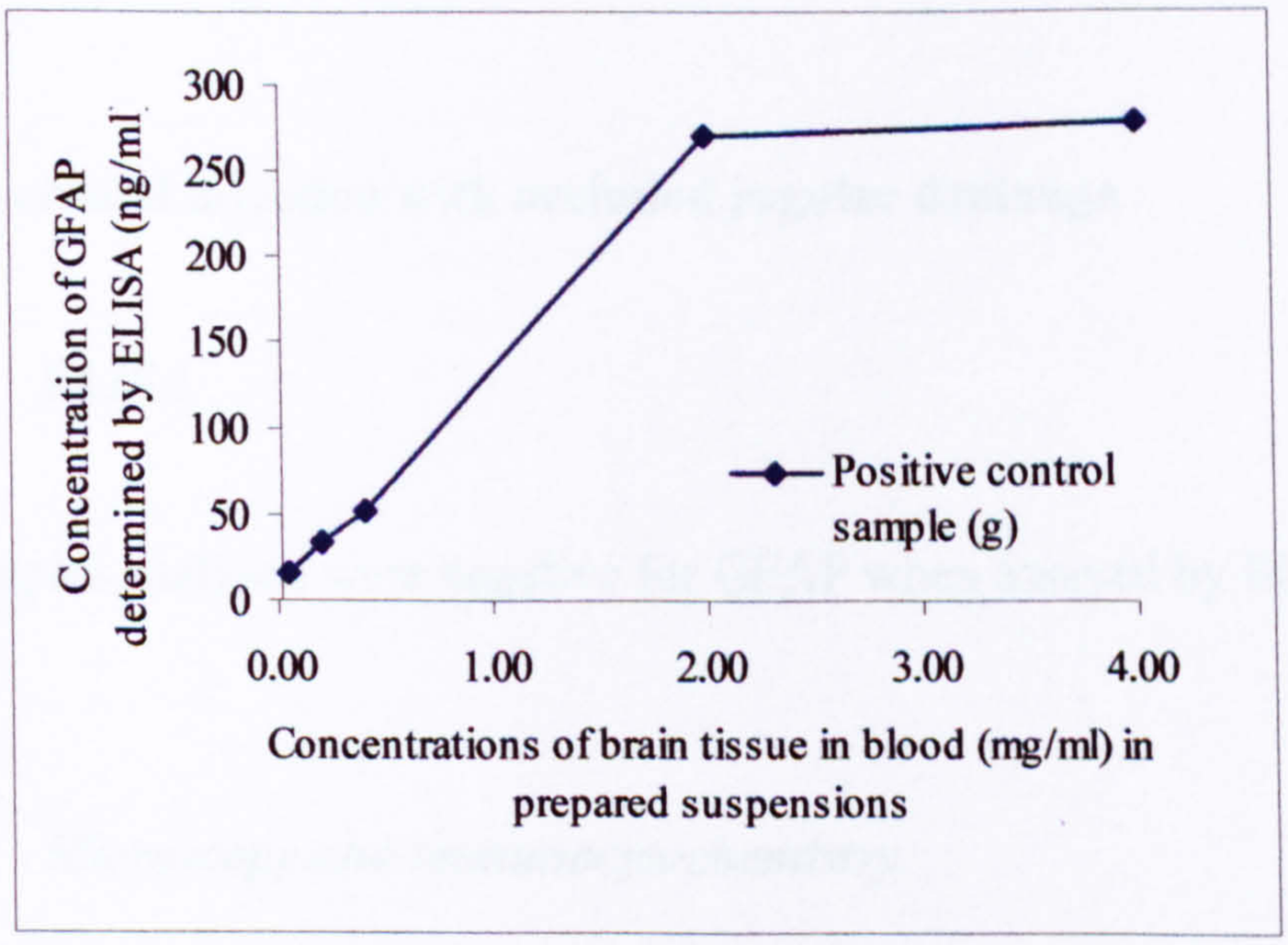


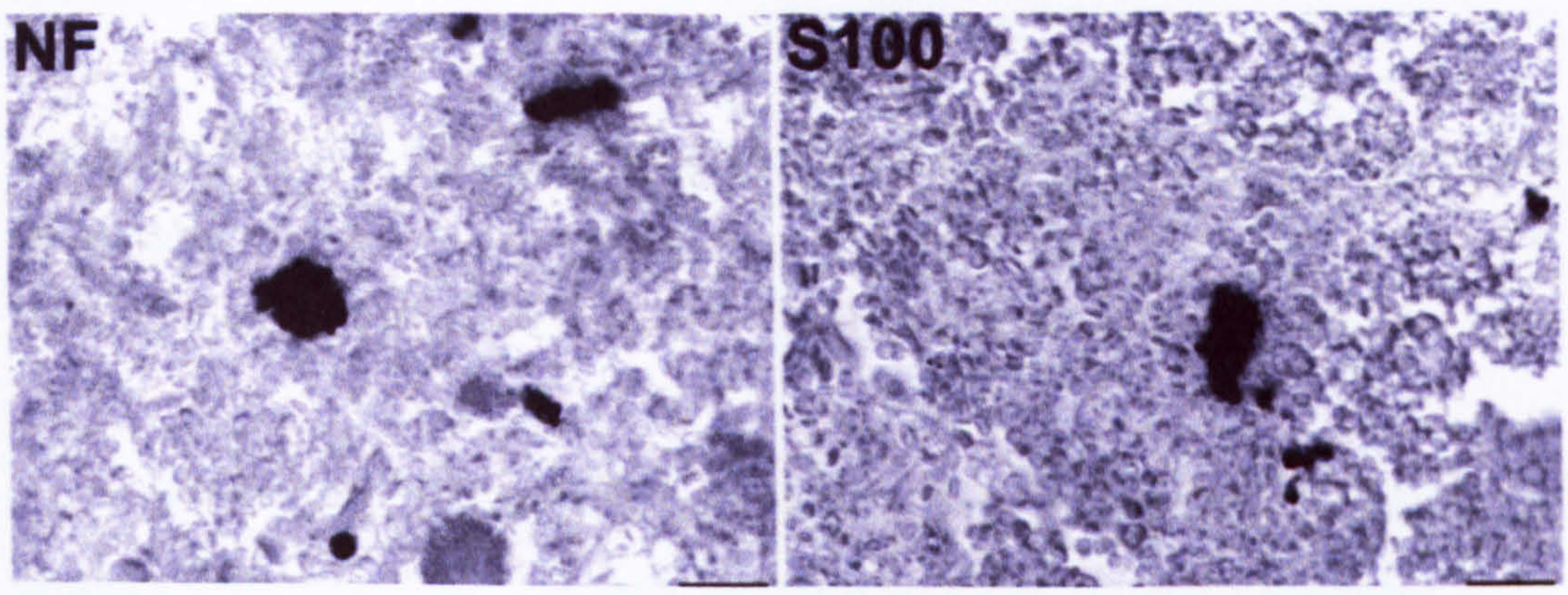

Figure 3.10 Concentrations of GFAP detected by ELISA in positive control samples [S (g)] prepared by the addition of known amounts of brain tissue to citrated blood samples



#### 3.1.4.6.2 *Microscopy and immunocytochemistry*

Blood samples from two (2/11) animals S (h) and S (k) yielded Cytoblock sections that contained material immunopositive for both S100β and neurofilament protein. None of the other blood samples were positive for either antigen on microscopy and immunocytochemistry.

Figure 3.11 Brain tissue fragments (black) in Cytoblocks prepared from aortic ovine blood samples stained with neurofilament and S-100β proteins




Bar=20μm

### 3.1.4.7 Intra-cranial injection with occluded jugular drainage

#### 3.1.4.7.1 *ELISA*

All aortic samples analysed were negative for GFAP when assayed by ELISA.

#### 3.1.4.7.2 *Microscopy and immunocytochemistry*

All aortic samples analysed were negative for brain tissue fragments.

### 3.1.4.8 Organ sampling following injection of brain tissue suspension and stunning

#### 3.1.4.8.1 *ELISA*

##### 3.1.4.8.1.1 *Organs*

All samples analysed by ELISA gave OD levels similar to background readings however unusually high background levels of GFAP were obtained from all samples making identification of any possible positives difficult

##### 3.1.4.8.1.2 *Blood*

Analysis of post-stun samples indicated elevated levels of GFAP in the jugular return of all samples

Table 3.14 Concentrations of GFAP (ng/ml) detected by ELISA in jugular blood samples from sheep after stunning and injection of brain tissue suspension

| Sample time (s) | Sheep 1 | Sheep 2 | Sheep 3 | Sheep 4 | Sheep 5 |
|---|---|---|---|---|---|
| Pre-stun sample | nd | nd | nd | nd | nd |
| 10 | 92.4 | 5.6 | 7.9 | 75.1 | 24.0 |
| 20 | 147.9 | 5.3 | 10.1 | 12.3 | 58.3 |
| 30 | 5.7 | 5.2 | 11.5 | nd | 2.4 |
| 40 | nd | 5.4 | 9.9 | nd | 70.9 |
| 50 | nd | nd | 3.7 | nd | 1.9 |
| 60 | nd | nd | 8.4 | nd | 30.3 |

Nd=not detectable by ELISA

#### 3.1.4.8.2 *Microscopy and immunocytochemistry*

Brain tissue was not detected in any of the tissue samples examined by microscopy and immunocytochemistry.

### 3.1.4.9 Validation

#### 3.1.4.9.1 *Cattle*

##### 3.1.4.9.1.1 *ELISA*

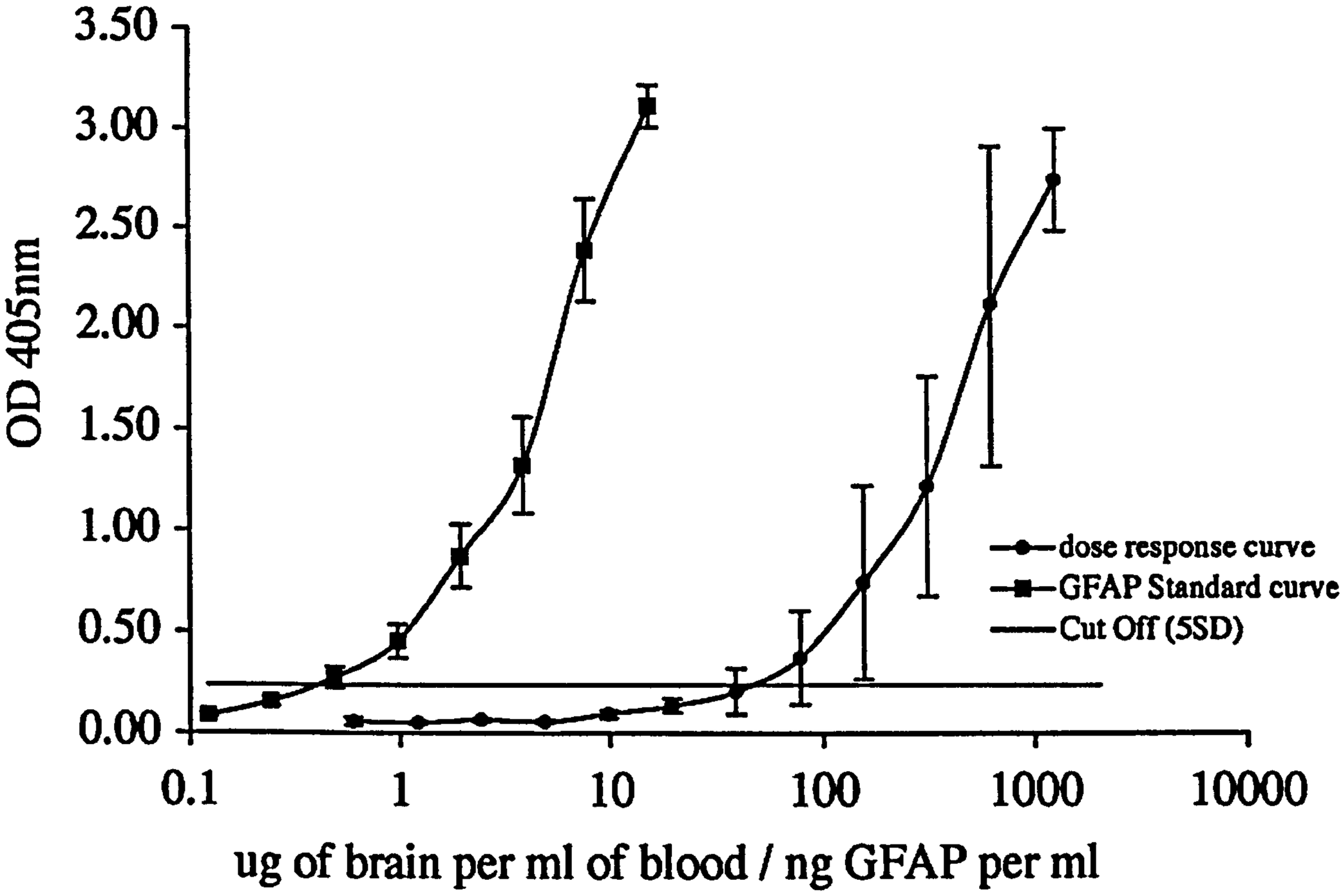
The ELISA readings obtained from the assay of the two-fold serial dilution of brain tissue in blood generated a curve that was parallel to that of the recombinant GFAP standard (Roche) (Figure 3.12). This indicates that CNS tissue can be detected and quantified when diluted in blood using the described methods.

The minimum detection limit of brain tissue in blood was found to be between 39µg/ml-156µg /ml (wet weight brain per ml blood). The minimum detection limit of GFAP was 0.5ng/ml. The actual cut-off point used to define a positive sample was higher at 1ng/ml or 0.05ng/well which correlates well with previous detection limits of GFAP as quoted in other studies (Helps et al., 2002).

The inter-assay co-efficient of variation between ten positive samples repeated (in duplicate) on two separate occasions was ≈ 17%.

The intra-assay co-efficient of variation between eight identical dilutions of the GFAP standard was ≈ 13%

Figure 3.12 Graph showing standard curve for the GFAP ELISA and a parallel recovery curve of dilutions of exogenously added bovine brain tissue in bovine blood




3.1.4.9.1.2 *Microscopy and immunocytochemistry*

The limit of detection of the analysis varies from 312µg/ml to 9.8µg/ml. All five dilutions were detectable down to a dilution of 312µg/ml, in four series dilutions to 39µg/ml and in a single dilution down to a minimum detectable dilution of 9.8µg/ml. The negative control samples were all correctly identified as negative.

Table 3.15 Positive bovine samples detected by microscopy and immunocytochemistry for determination of the limit of detection of the analytic method

| Brain concentration (mg/ml) | Sample number | | | | | |
|---|---|---|---|---|---|---|
| | 1-15 | 16-30 | 31-45 (-)control | 46-60 | 61-75 | 76-90 |
| 10.00 | + | + | - | + | + | + |
| 5.00 | + | + | - | + | + | + |
| 2.500 | + | + | - | + | + | + |
| 1.250 | + | + | - | + | + | + |
| 0.6250 | + | + | - | + | + | + |
| 0.3125 | + | + | - | + | + | + |
| 0.1560 | + | + | - | nd | nd | + |
| 0.0780 | nd | + | - | nd | nd | + |
| 0.0390 | + | + | - | nd | + | + |
| 0.0195 | nd | nd | - | + | nd | nd |
| 0.0098 | nd | nd | - | nd | nd | + |
| 0.0049 | nd | nd | - | nd | nd | nd |
| 0.0024 | nd | nd | - | nd | nd | nd |
| 0.0012 | nd | nd | - | nd | nd | nd |
| 0.0006 | nd | nd | - | nd | nd | nd |

+ = Samples positive by microscopy and immunocytochemistry

Nd = not detectable

- = negative samples

### 3.1.4.9.2 *Sheep*

#### 3.1.4.9.2.1 *ELISA (blood)*

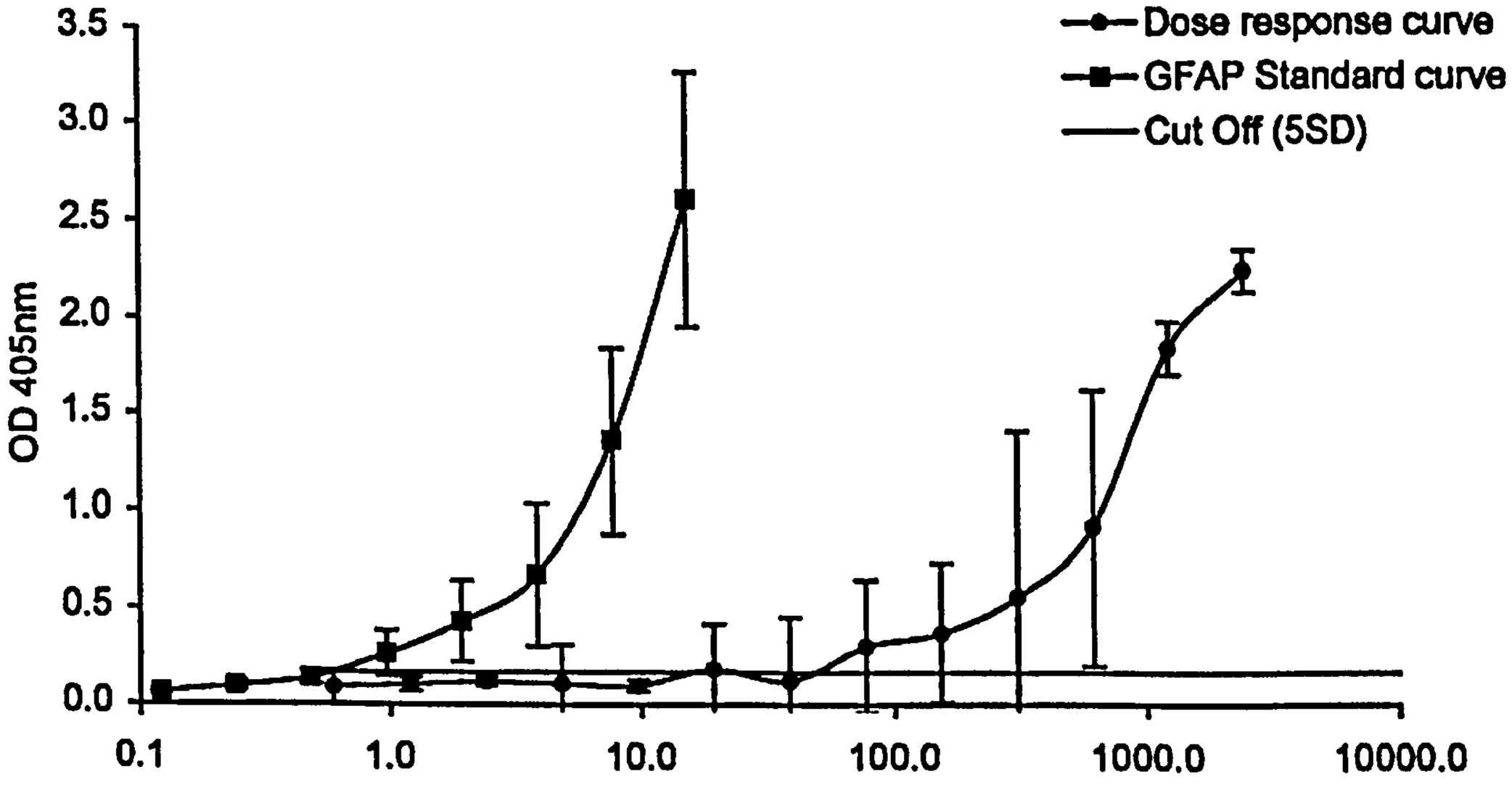
The ELISA readings obtained from the assay of the two fold serial dilution of brain tissue in blood generated a curve that was parallel to that of the recombinant GFAP standard. This indicates that CNS tissue can be detected and accurately quantified when diluted in blood by this method. The lower detection limit of GFAP was approximately 0.5ng/ml although samples with a concentration below 1ng/ml were considered negative. The lower detection limit of brain tissue in blood was found to be between

39µgml -156µg /ml (brain wet weight/ml blood). The inter-assay co-efficient of variation between ten positive samples repeated (in duplicate) on two separate occasions was ≈ 17%. The intra-assay co-efficient of variation was ≈ 13%

The cut-off value for a positive sample was defined as five times the standard deviation plus the background level. The background was calculated from a random selection of pre-stun samples assuming a normal distribution of the OD values obtained by spectrophotometric reading of the ELISA plates [mean pre-stun OD= 0.0736 where n=11, SD=0.0174, cut-off = 0.16 (0.074+5*0.0174)

GFAP was consistently detected in all samples above the limit of detection and was absent in all negative control samples.

Figure 3.13 Graph showing standard curve for the GFAP ELISA and a parallel recovery curve of dilutions of exogenously added ovine brain tissue in ovine blood



### 3.1.4.9.2.2 *ELISA (Tissues)*

GFAP was detected in all three samples but was absent from the negative control samples.

Table 3.16 Concentration of GFAP detected by ELISA in bovine lymph nodes previously injected with suspensions of brain tissue

| Quantity of brain tissue injected into each lymph node | GFAP (ng/ml) |
|---|---|
| 100mg | 9.7 |
| 200mg | 11.5 |
| 400mg | 20.75 |
| 0.00 (negative control) | nd |
| 0.00 (negative control) | nd |
| 0.00 (negative control) | nd |

nd=not detectable

### 3.1.4.9.2.3 *Microscopy and immunocytochemistry*

The limit of detection of the analysis varies from 312µg/ml to 9.8µg/ml. All five dilutions were detectable down to a dilution of 312µg/ml in two series dilutions to 39µg/ml and in a single dilution down to a minimum detectable dilution of 9.8µg/ml. The negative control samples were all correctly identified as negative.

Table 3.17 Positive ovine samples detected by microscopy and immunocytochemistry for determination of the limit of detection of the analytic method

| Brain concentration (mg/ml) | Sample number | | | | | |
|---|---|---|---|---|---|---|
| | 1-15 (negative control) | 16-30 | 31-45 | 46-60 | 61-75 | 76-90 |
| 10.00 | - | + | + | + | + | + |
| 5.00 | - | + | + | + | + | + |
| 2.500 | - | + | + | + | + | + |
| 1.250 | - | + | + | nd | + | + |
| 0.6250 | - | + | + | + | nd | + |
| 0.3125 | - | + | + | + | + | + |
| 0.1560 | - | + | nd | nd | + | nd |
| 0.0780 | - | nd | nd | nd | nd | nd |
| 0.0390 | - | + | nd | nd | + | nd |
| 0.0195 | - | nd | nd | nd | nd | nd |
| 0.0098 | - | nd | nd | nd | nd | + |
| 0.0049 | - | nd | nd | nd | nd | nd |
| 0.0024 | - | nd | nd | nd | nd | nd |
| 0.0012 | - | nd | nd | nd | nd | nd |
| 0.0006 | - | nd | nd | nd | nd | nd |

nd = Not detectable by the analytic method

+ = brain tissue was detected

- = no brain tissue present

## 3.1.5 Discussion

The frequency with which brain tissue fragments enter the venous circulation of livestock following the use of mechanical stunning methods is of considerable importance in assessing the safety of these techniques. Since the emergence of BSE in cattle and of vCJD in humans, control measures have been implemented to restrict all potential routes by which transmission of BSE to humans could occur. The use of captive bolt guns for stunning livestock prior to slaughter has been suggested as a risk

factor for the contamination of carcasses with tissues of potentially high BSE infectivity (Garland, Bauer & Bailey, 1996; Anil et al., 1999; 2001). The findings of these preliminary observations and experiments highlighted the need for an in-depth investigation of the use of captive bolt guns and the frequency and extent of contamination of carcasses with brain tissue. In addition, this work suggested the need for a review of stunning methods currently in use in the UK.

In the experiments described in this thesis, the risks associated with the use of stunning methods currently in use or that are authorised for use in the UK, were comprehensively investigated using a variety of applied techniques. The results of analyses of blood samples collected from cattle and sheep after stunning indicate that all four of the captive bolt guns examined (See 2.1.4), can cause entry of brain tissue fragments into the venous circulation. The range of frequencies of brain tissue embolism determined compares with figures reported in a study of brain tissue embolism after severe head trauma in humans (Collins & Davis, 1994). The similarity in the frequency ranges reported in this and other related studies (Garland, Bauer & Bailey, 1996), despite differences in the type and causes of head trauma, suggests that the occurrence of brain tissue embolism may need to be considered after use of any stunning method that causes severe skull and brain trauma.

Extrapolation of the frequency of brain tissue embolism determined in this experiment (2 to 4%) to the total number of cattle slaughtered annually in the UK (GLIPHA, 2001) suggests that between forty and eighty thousand carcasses may risk contamination after captive bolt gun stunning. Furthermore, consideration of historical data on the numbers of cattle slaughtered after the emergence of BSE suggests that a significant number of carcasses may have been contaminated by this route between 1985 and 1995, before effective control measures for BSE were instituted.

The comparatively high frequencies of brain tissue embolism detected in the sheep study must strengthen arguments for the blanket use of alternative stunning methods in this species. Experimentally induced BSE in sheep is known to result in widespread infectivity in the carcass in comparison to cattle in which nearly all infectivity is restricted to tissues currently designated as SRM (Jeffrey et al., 2001). In view of this finding, additional BSE contamination through brain tissue dissemination in an already BSE infected carcass might significantly increase the risk to the consumer of ingesting

an infective dose. The high frequencies of brain tissue emboli detected in sheep as compared to cattle samples may indicate a fundamentally greater risk of brain tissue embolisation in sheep after CBG stunning. The smaller sheep brain might be expected to suffer proportionately greater trauma and be subjected to greater intra-cranial pressures after stunning than the larger cattle brain, resulting in higher frequencies of brain tissue emboli. Alternatively, this finding may relate to differences in the method of sample collection and processing. The smaller body weight of sheep along with the commensurate smaller blood volumes collected during sampling may have resulted in an overall higher detection rate of brain tissue emboli in sheep samples.

Statistical comparison of the frequency of brain tissue emboli detected in each species indicated no significant difference between each CBG group examined. However, the small numbers of positive samples detected in the study does not allow reliable comparisons of each gun in cattle. A retrospective analysis of the data and calculation of the power of the test obtained from the cattle studies indicated only a 13% chance of detecting a difference between each sample group. Increasing each sample group size to 500, 1000 or 2000 animals would have increased the chances of detecting a difference between uses of each CBG to 46%, 75% and 96% respectively. Clearly, further studies examining greater numbers of cattle might reveal significant difference in risk between the use of the non-penetrating and penetrating guns. In the parallel sheep study, calculation of the power of the chosen sample size indicated a 37% chance of detecting a difference between stunning with each CBG. If the sample group size had comprised 500 or 1000 animals the chances of detecting a difference would have increased to 96% or 99.9% respectively. In light of the calculated powers of each experiment and irrespective of cost and logistical implications a group size of at least 2000 cattle and 500 sheep would have best increased the chances of detecting a difference between the use of each CBG in the respective species.

Non-penetrating captive bolt guns were previously thought unlikely to carry a risk of brain tissue embolisation (EC, 2001), although investigations of brain trauma after stunning indicated comparable or greater levels of brain tissue damage to that observed after penetrating CBG stunning (Farag, 2001). The finding in this study, of brain tissue fragments in samples from two cattle after the use of a non-penetrating CBG was unexpected and has cast doubt upon the use of these guns as a safer alternative to penetrating CBG stunning.

Brain tissue emboli were detected within a minute of blood collection, and in most cases were found in samples taken within thirty seconds of stunning. Given this time-frame in the real abattoir environment, the majority of emboli would have sufficient time to pass through the jugular veins and heart to challenge the pulmonary capillary filter before the jugular veins were severed and the circulation halted by cardiac arrest as a result of exsanguination. Stunning to sticking intervals vary among abattoirs but will generally lie in the range of thirty to ninety seconds.

The methods of isolating and detecting brain tissue emboli employed in these investigations were developed in preliminary studies that examined the occurrence of brain tissue emboli after CBG stunning in a small number of cattle and sheep (Anil et al., 1999; 2001; 2002). Prior to the development of these novel techniques, brain tissue emboli had been reported as an unexpected finding at post-mortem examination of cattle carcasses (Garland, Bauer & Bailey, 1996). The unreliability of investigations based solely upon post-mortem examination, was highlighted by a later investigation of neural embolism after stunning that failed to detect any brain tissue emboli in over two hundred cattle carcasses examined (Munro, 1997).

By capturing all the jugular venous blood draining from the head within the first minute of stunning, the methods utilised and described in this study should minimise the risk of overlooking brain tissue fragments disrupted by captive bolt gun stunning. Furthermore, this technique allows detection of brain tissue emboli in aortic blood samples. These small fragments and proteins would have already passed though the lungs and would not be detectable by post-mortem examination of the heart and lungs of carcasses.

The use of two analytic methods increased the chances of detecting emboli although examination of the results obtained revealed a level of discrepancy between samples identified as positive by each assay. This apparent disparity between the ELISA and analysis by microscopy and immunocytochemistry was not entirely unexpected in view of the findings from a previous related study that also found some disagreement in results of two analyses (Anil et al., 2002). Sampling variance of the isolated buffy coat fraction during processing may have resulted in an uneven distribution of brain tissue fragments between samples sent for analysis. Furthermore, each analytic method examined less than one percent of the blood collected and consequently carried a risk of

over-looking some positive samples. It is also possible that elevated levels of GFAP may have been present in some samples without the presence of identifiable fragments of brain tissue. The negative findings by ELISA from samples in which brain tissue fragments were observed by microscopy may relate to enzymatic degradation of GFAP in some positive samples during storage (Missler et al., 1999).

The experiments conducted to establish the performance of the two analytic methods using prepared brain in blood suspensions, suggested that both will correctly identify samples containing brain tissue material. Prepared tissue samples indicated that the ELISA could also detect brain tissue contamination in peripheral organs. However, samples obtained from a sheep after stunning and injection of brain tissue suspension indicated a high background level of GFAP that interfered with detection of brain tissue contamination. These findings indicated the need for development of an alternative method of detecting brain tissue contamination of organs.

In a study of head trauma in humans, some brain proteins including GFAP were found to be consistently elevated in all blood samples collected and analysed (Mussack et al., 2003). On the contrary, in this investigation relatively few samples demonstrated elevated levels of GFAP despite all animals being stunned prior to sample collection. The reason for this anomaly may relate to the reaction of astrocytes to trauma. Following head trauma, astrocytes are known to react by producing and releasing large quantities of GFAP into the circulation (Petzold et al., 2004). Although this process occurs quickly, it is not immediate and would not be a factor in these experiments of captive bolt gun stunning. In contrast, blood samples in human trials cannot be collected immediately after head trauma, allowing increased production of GFAP and explaining consistently elevated serum GFAP levels in all samples collected.

Electrical stunning offers an alternative method of stunning, the use of which is well established for use in sheep in the UK, and which may see wider application for cattle stunning in the future. By combining a macroscopic study of cattle brains with analysis of blood samples taken after stunning, this experiment has confirmed predictions that electrical stunning is unlikely to cause brain tissue fragments to enter the circulation.

The experiments in which aortic blood was sampled following injection of brain tissue suspensions into the jugular return were successful in confirming the potential of brain

tissue fragments and proteins to penetrate the pulmonary capillary filter. The findings of these investigations have given the first experimental evidence that brain tissue emboli created by captive bolt gun stunning are likely to enter the systemic circulation and be carried to edible parts of the carcass. Furthermore, the size of emboli detected in some ovine aortic samples indicates the size of brain tissue fragments that are capable of penetrating the pulmonary capillaries. The time with which such fragments entered the systemic circulation was well within the period that would occur between stunning and sticking in a commercial abattoir. In further studies, the use of larger sampling populations might enable more accurate estimation of the proportion of stunned animals in which this is likely to occur.

## 3.2 ANATOMICAL INVETSIGATION OF VENOUS CEREBRAL DRAINAGE

### 3.2.1 Introduction

The technique used in these experiments follows that described by the University of Bristol anatomy department (Mr. Steve Gaze-personal communication) where the method has been used to produce a variety of casts of organ vasculature for demonstration purposes in the department. The technique however has not previously been applied to the cerebral venous sinuses and vessels of the vertebral plexus of cattle and sheep.

These casts may be useful in identifying the vessels that are likely to be traumatised by the captive bolt at stunning and also for estimating the vessel diameters that may suggest the size of emboli, which may enter the circulation following application of stunning.

### 3.2.2 Aims

- To investigate all venous drainage routes from the head that may contribute towards carriage of brain tissue emboli following the use of captive bolt gun stunning in sheep and cattle.

### 3.2.3 Methodology

#### 3.2.3.1 Resin casting

##### *3.2.3.1.1 Bovine 4 weeks*

The specimen was obtained from a four-week old Holstein male calf of approximately 60-kg live weight. Injection of resin was made into the left jugular vein only as the right jugular vein had retracted into the subcutaneous tissues and could not be located. A total quantity of 60- ml of resin was injected although resin was not observed at the cut-end of the neck. Increasing back-pressure on the syringe prevented further injections of resin.

*3.2.3.1.2 Adult bovine*

This specimen was obtained from an OTMS abattoir. Injections of resin into both jugular veins was attempted although injection on the left side was stopped after injection of approximately 50-ml of resin was injected due to increased back-pressure on the syringe. The remaining quantity of resin was injected into the right jugular vein only. In total 300-ml of resin was injected before leakage of resin was observed from the cut-ends of the vertebral veins.

3.2.3.1.3 *Adult ovine*

Injections of approximately 30-ml of resin were made into each jugular vein before resin was observed at the cut-ends of the vertebral veins.

### 3.2.3.2 Dissection of adult ovine head and neck

A fresh adult sheep cadaver was obtained from the Langford site abattoir (University of Bristol) following the use of electrical stunning and slaughter by exsanguination. The head and neck were separated from the carcass at the level of the fourth cervical vertebrae.

## 3.2.4 Results

### 3.2.4.1 Resin casting

3.2.4.1.1 Bovine 4 weeks

The dorsal cerebral sinus system (dorsal sagittal sinus, transverse sagittal sinus, confluens sinuum, deep cerebral vein) of the specimen demonstrated good perfusion with resin on both sides despite having injected only through the left jugular vein. The basilar system of sinuses and the vertebral plexus demonstrated incomplete perfusion with resin with no part of the left basilar plexus visible and only a short length (30-cm)

of the vertebral plexus present on the left side of the specimen. The length of vertebral plexus visible demonstrates vessels of significant dimensions (Table 3.16) that continue as the basilar sinus plexus cranial to the foramen magnum.

Figure 3.14 Resin cast of cerebral venous drainage of calf four weeks into the maceration process

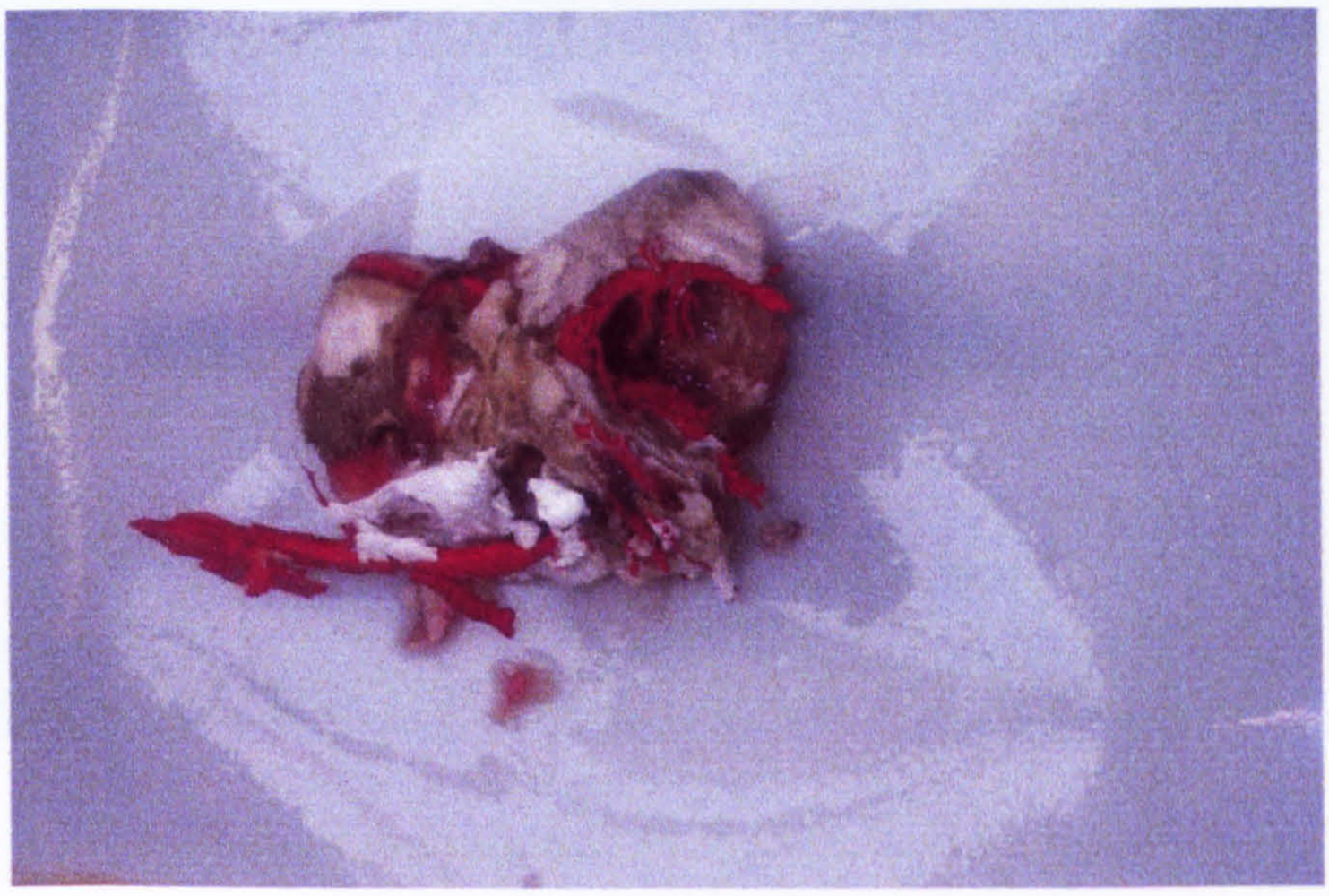
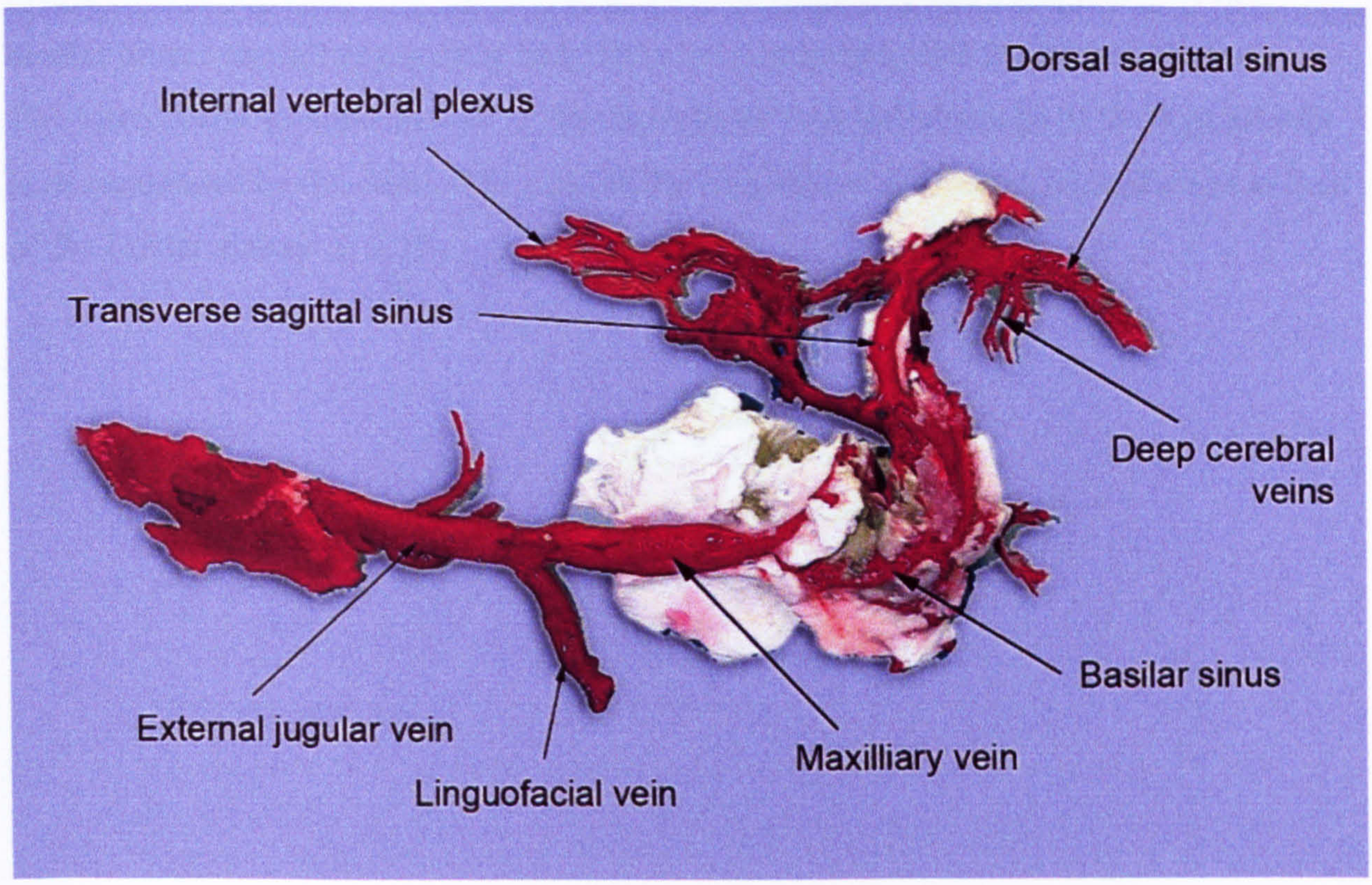


Table 3.18 Measured diameters of the cast of some veins of the head and neck in the calf specimen

| Vessel | Diameter (mm) |
|---|---|
| Dorsal sagittal sinus | 7 |
| Transverse sagittal sinus | 4-6 |
| Deep cerebral vein | 1 |
| Basilar sinuses | 9 |
| Internal vertebral plexus | 3-6 |
| Jugular vein | 11 |

Figure 3.15 Resin cast of cerebral venous drainage of calf




*3.2.4.1.2 Adult bovine*

Resin injection resulted in excellent perfusion of the cerebral sinuses and the internal vertebral plexus although efficient perfusion appears to have occurred only from the right jugular vein. A constriction of the left jugular vein at the level of the angle of the jaw suggests that comparatively less resin was injected on this side. The left side of the cast is incomplete although the basilar plexus appears symmetrical. The internal vertebral plexus consists of a pair of longitudinal vessels interconnected by transverse interconnecting anastomoses. The longitudinal vessels are segmentally arranged with convexities interconnected with areas in which the vessels approach and appear to merge. The diameter of the vessels decreases slightly over the length of the cast corresponding to the edge of the fourth cervical vertebrae at which point the cast ends. Cranially the ventral vertebral plexus merges with the basilar sinus which itself gives off the orbital veins that are only visible on the right side of the cast. The vertebral vein is also visible running dorsal and lateral to the plexus on the right side of the cast. The diameter of the vertebral vein appears constant up to the level of C2 at which point the vein branches and forms a small plexus with branches off from the internal vertebral

plexus. The cerebral sinuses are demonstrated on the right side of the cast although some vessels including the greater part of the dorsal sagittal sinus was lost during preparation of the cast. The cast demonstrated the large size and complexity of the basilar plexus as well as the clear and extensive connection with the vertebral plexus. The intercavernous sinus as well as the cup-shaped vascular drainage of the hypophysis is demonstrated by the resin. The ratio of the diameter of the dorsal sagittal sinus to that of the basilar sinuses is 5.7%.

Table 3.19 Measured diameters of the cast of some veins draining the head of an adult bovine

| Vessel | Diameter (mm) |
|---|---|
| Dorsal sagittal sinus | 2 (width) x 8 (depth) |
| Transverse sagittal sinus | 8 |
| Confluence of sinuses | 3 |
| Dorsal occipital plexus | 3 |
| Deep cerebral vein | 4 |
| Basilar sinuses | 35 |
| Condylar sinus | 9 |
| Temporal sinus | 8 |
| Inter-cavernous sinus | 2 |
| Cavernous sinus | 12 |
| Emissary vein of foramen orbitorotundum | 10 |
| Dorsal cerebral vein | 2 |

Table 3.20 Measured diameters of the cast of some veins of the neck of the adult bovine specimen

| Vessel | Diameter (mm) |
|---|---|
| Internal vertebral plexus (C1-C4). | C1= 7<br>C2= 6<br>C3= 5<br>C4= 4 |
| Basivertebral veins | 2 |
| Transverse anastomoses | 10 |
| Vertebral vein | 8 |
| Spinous branch | 2 |
| Jugular vein | 21 |

Figure 3.16 Bovine head and neck cast specimen during maceration in sodium hydroxide solution

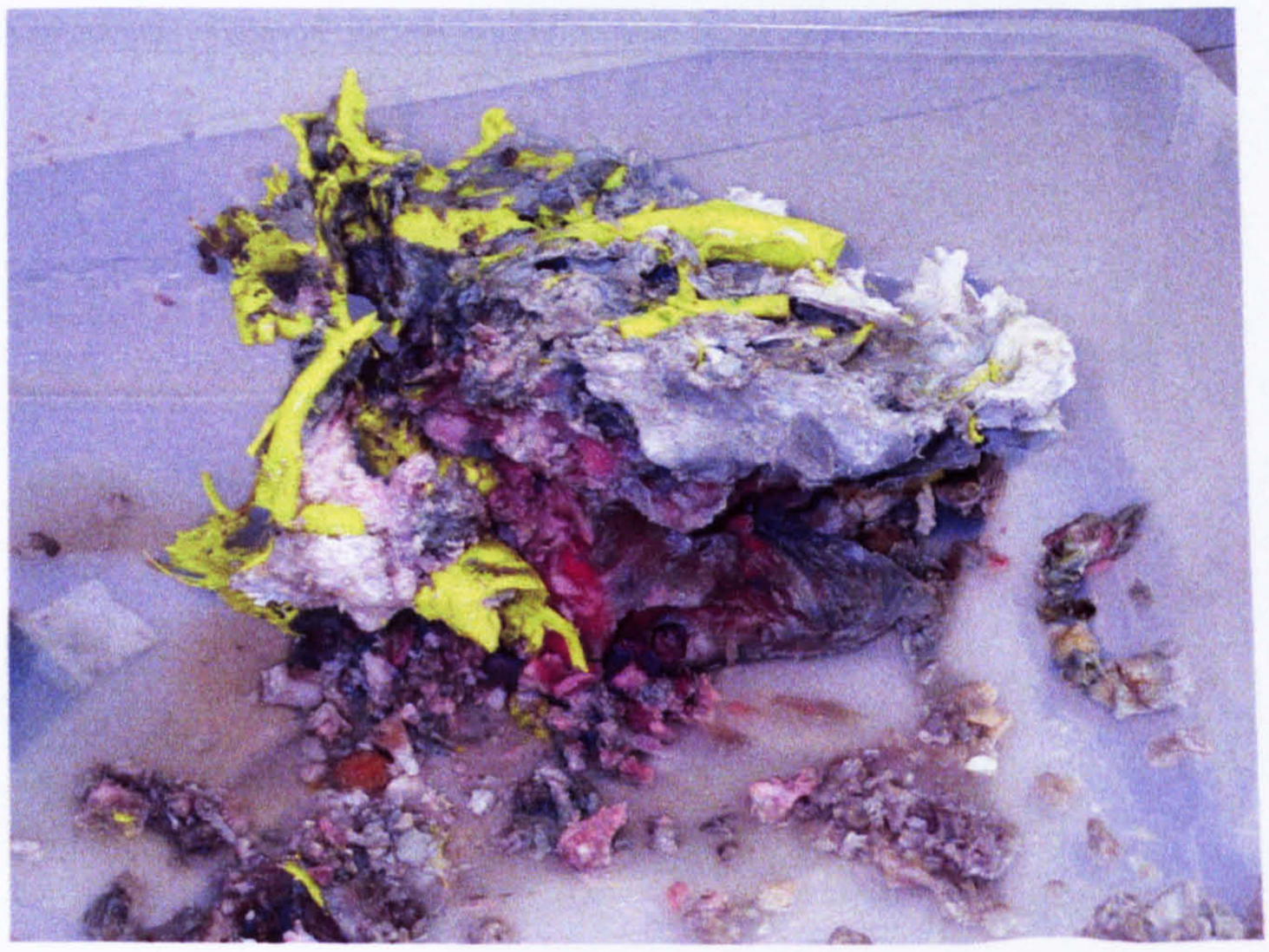


Figure 3.17 Lateral view of resin cast of bovine cerebral sinuses and vertebral plexus

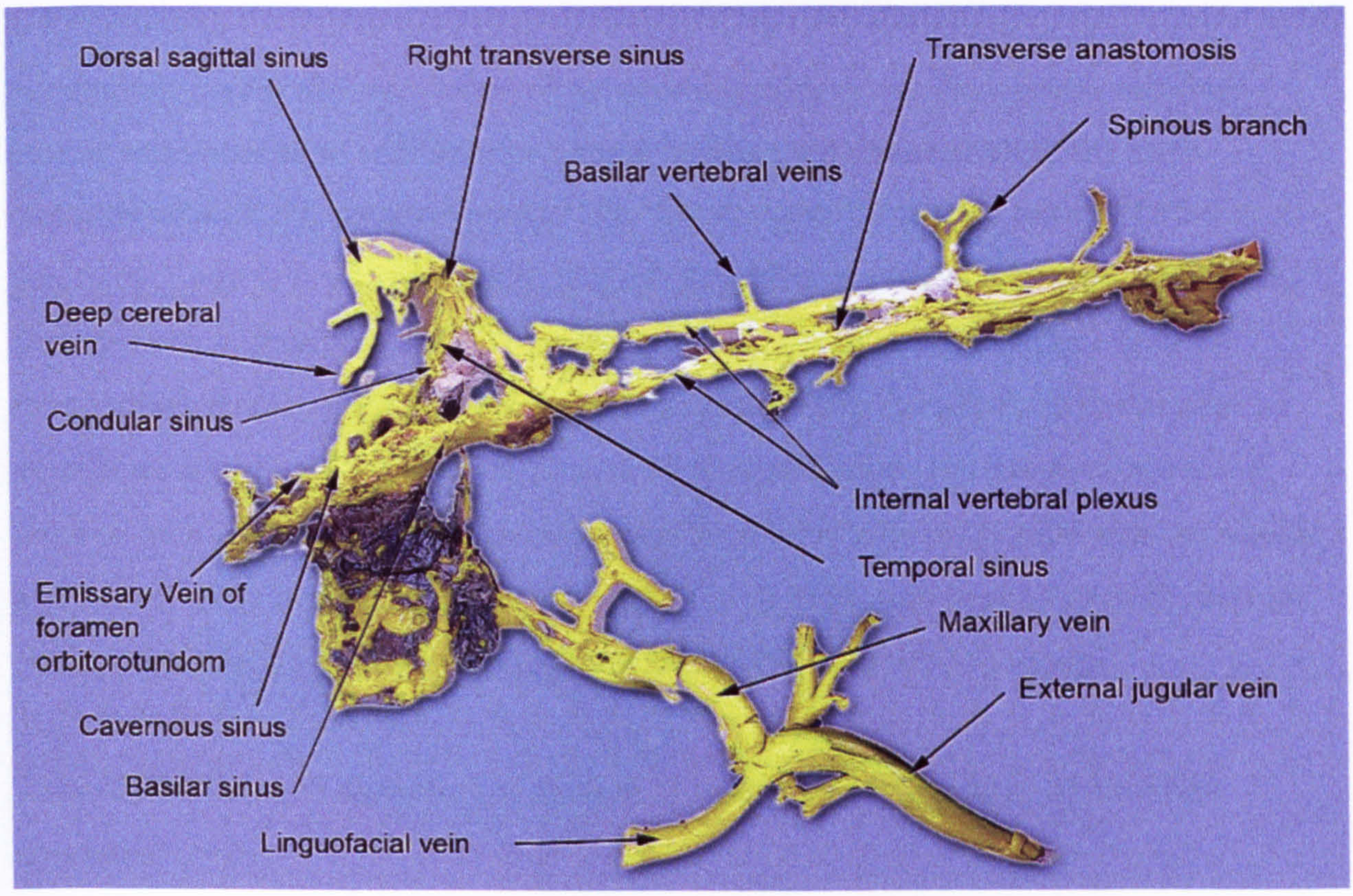
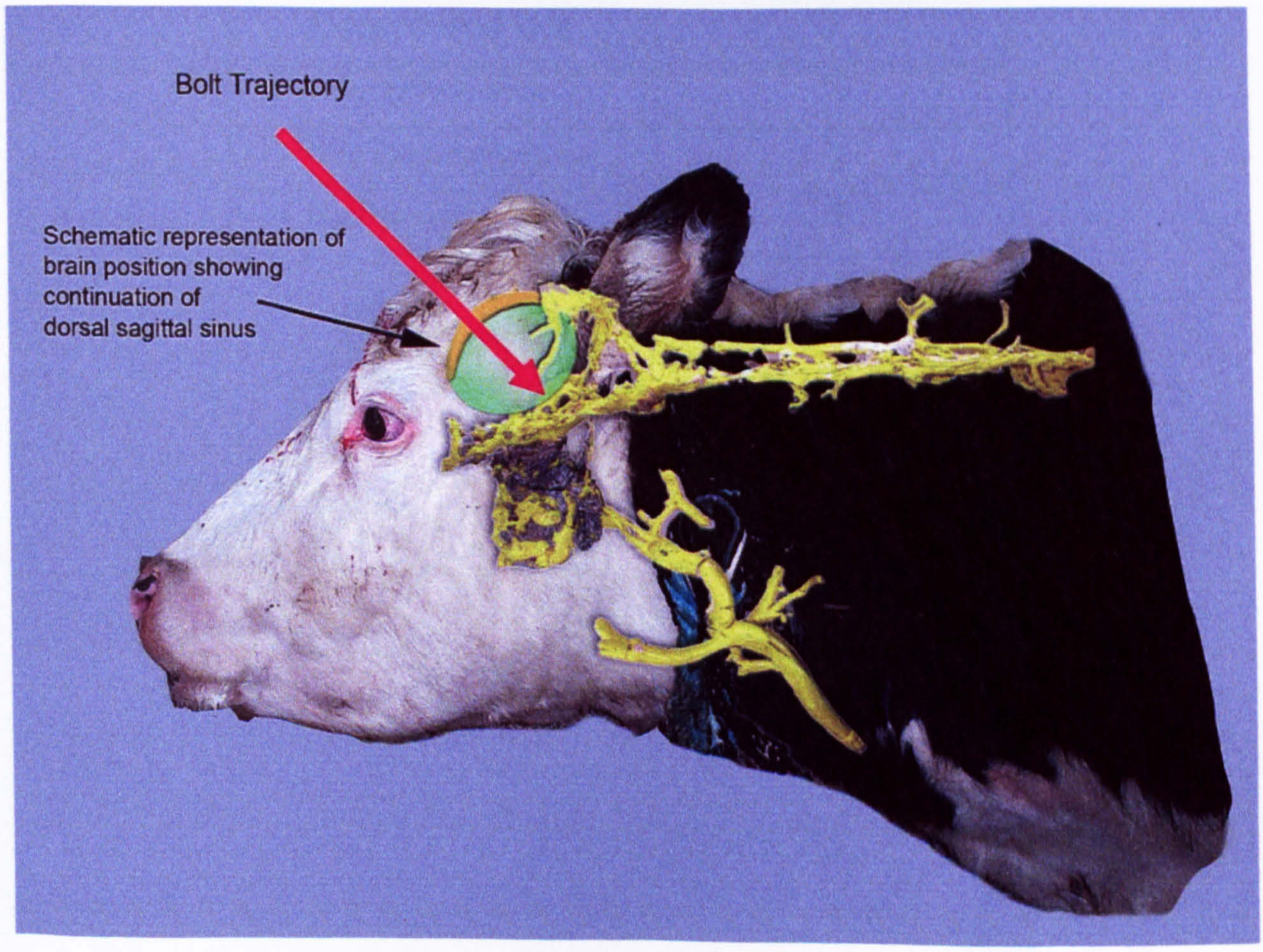



Figure 3.18 Lateral view of the resin cast of cerebral sinuses and vertebral plexus superimposed on an adult bovine head indicating the relative positions of vessels and of the bolt trajectory



### 3.2.4.1.3 Adult ovine

A complete cast of the vasculature of the cerebral sinuses and the internal vertebral plexus was obtained in addition to an intact jugular vein demonstrating the inter-connections with the vertebral plexus. The dorsal sagittal sinus is a vessel of 50-mm in length by 2-mm at it is widest width that follows the curvature of the brain. The basilar sinus plexus is largely absent in the specimen although caudally it merges with the longitudinal vessels of the internal vertebral plexus. These longitudinally- directed vessels are intact until the level of C5 and demonstrate wide inter-connections at the level of each vertebra. Two veins connecting the plexus with the jugular vein are visible at the level of C1 and C4. The external vertebral plexus is not demonstrated by the cast perhaps reflecting a failure of the resin to perfuse this network of vessels.

Table 3.21 Measured diameters of the cast of some veins of the head of the ovine specimen

| Vessel | Diameter (mm) |
|---|---|
| Dorsal sagittal sinus | 2 |
| Transverse sagittal sinus | 3 |
| Deep cerebral vein | 1 |
| Cavernous sinus | 10 |
| Inter-cavernous sinus | 3 |
| Basilar sinus plexus | 5 |

Figure 3.19 Lateral view of the resin cast of the ovine cerebral sinuses

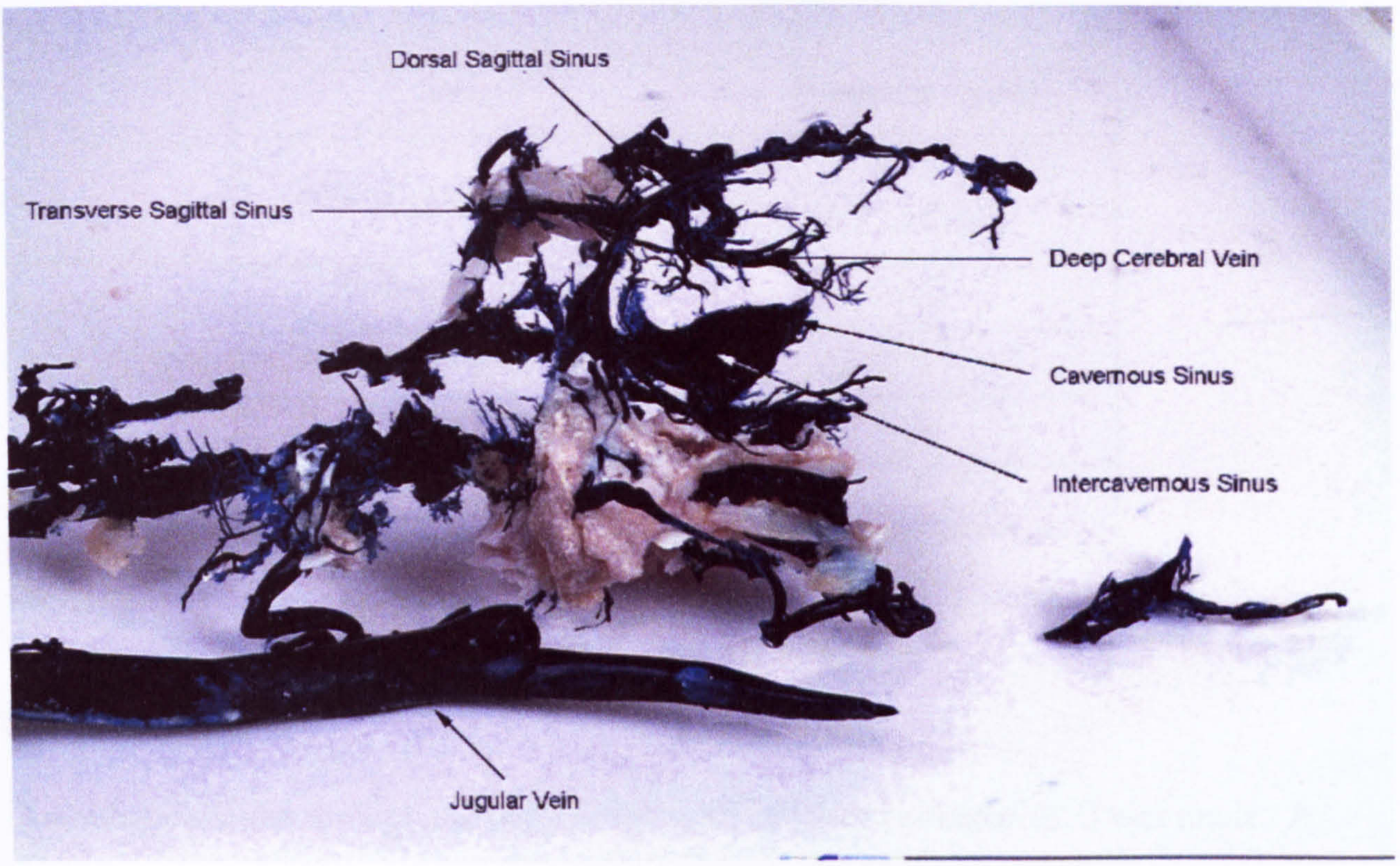



Figure 3.20 Lateral view of the cast of the ovine cerebral sinuses and cerebral drainage veins

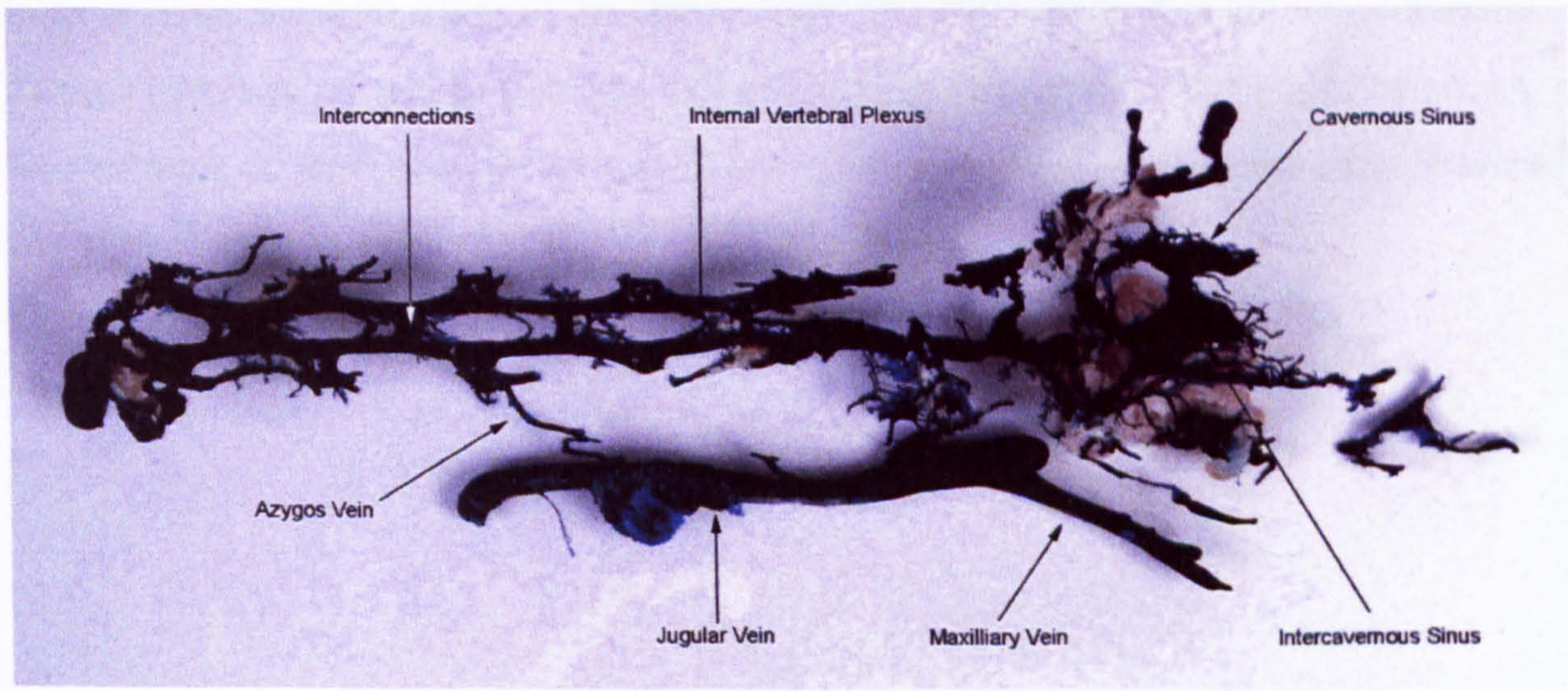


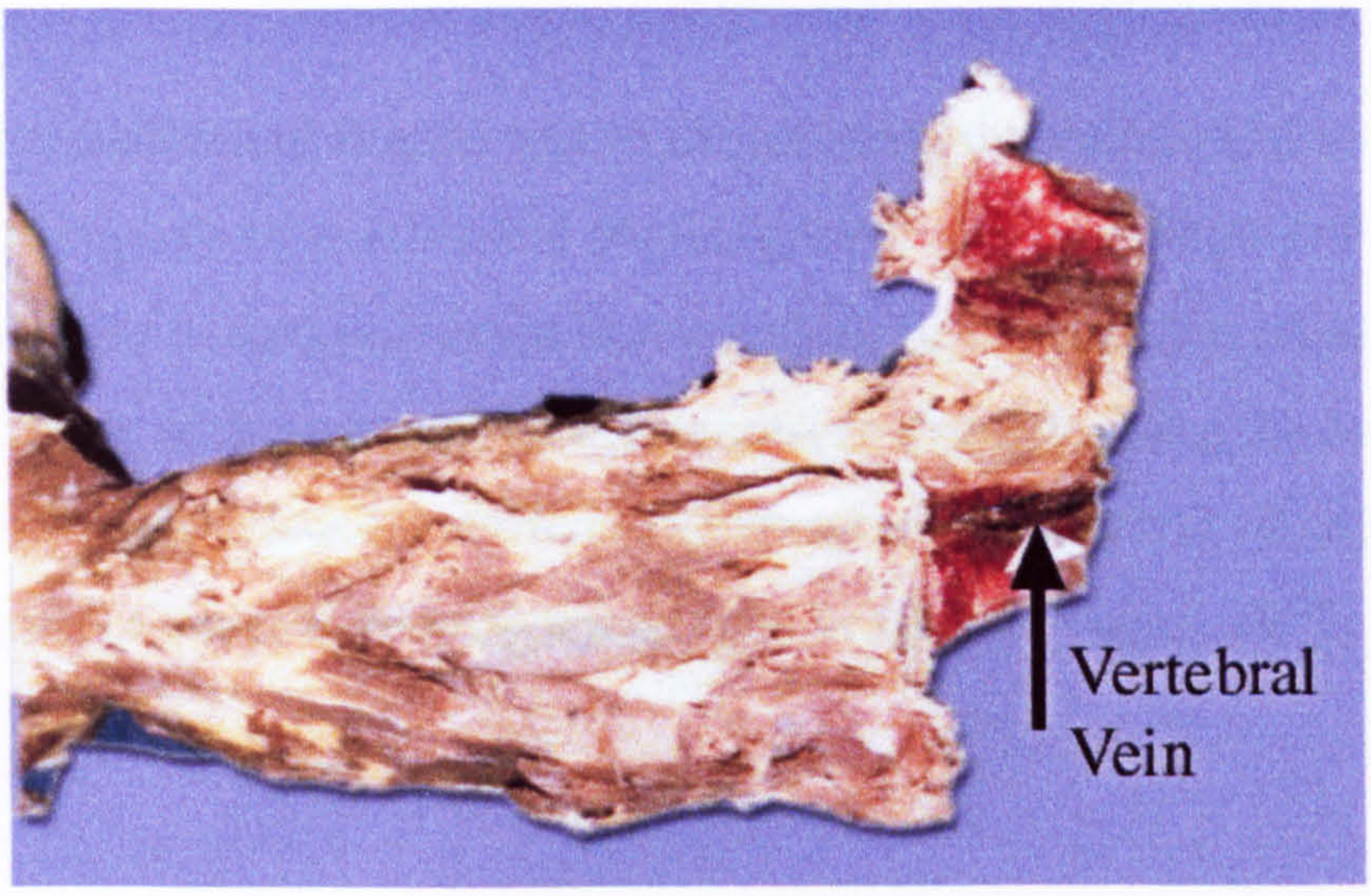
Table 3.22 Measured diameters of the cast of some veins of the neck of the ovine specimen

| Vessel | Diameter (mm) |
|---|---|
| Internal vertebral plexus | C1=6<br>C2 to C4=4 |
| Basivertebral veins | ≤ 1 |
| Transverse anastomoses | 5-12 |
| Spinous branch | 1 |
| Jugular veins | 9 |

### 3.2.4.2 Dissection of adult ovine head and neck

An oblique section through the body of the third cervical vertebrae (C3) was made. A large (4-mm diameter), valve-less vessel was exposed running close to and lateral to the spinal cord within a canal. The vessel was in a ventrolateral position relative to the body of the vertebrae. The vein on the other side was not exposed. Due to the significant size and position, this vein was identified as the vertebral vein (*V. vertebralis)*. Several pictures were taken of the exposed vessel. Removal of overlying bone exposed the dura mater of the spinal cord. The dura mater was cut longitudinally along a length of several centimetres and reflected to expose the underlying tissues. A fine network of superficial blood vessels was visible on the surface of the cord. Several photographs were taken.

Figure 3.21 Dissected sheep neck indicating the position of the vertebral vein




### 3.2.5 Discussion

The cerebral drainage pathways of humans and animals have been investigated by anatomical study since the 16th Century (Worthman, 1956). The descriptions of the vertebral venous plexus in the literature indicate an essentially similar system of spinal veins in humans and animals. Although the vertebral venous plexus is known to comprise a prominent network of blood vessels, the jugular veins are still considered as the major venous outflow despite evidence that this may not always be the case (Epstein et al., 1970; Zippel et al., 2001). The functional role of the vertebral plexus has been investigated in terms of its physiological significance as a collateral route of venous return to the heart and as a vascular bypass during transient increases in thoracoabdominal pressure (Zippel, 2001). A pathological role of the plexus in transporting bacteria and neoplastic cells from the head to other areas of the body has been suggested (Batson, 1940).

Since the emergence of BSE and the realisation that brain tissue emboli created by mechanical stunning methods may cause contamination of the carcass with infected prions (Garland, Bauer & Bailey, 1996), all potential contributing routes of dissemination of emboli in the carcass should be investigated and evaluated. The anatomical features of the venous cerebral sinuses that permit entry of brain tissue fragments following stunning have not previously been considered. Furthermore, the

dimensions and extent of the vertebral plexus has not been investigated in relation to the size and potential for dissemination of brain tissue fragments in the carcass.

The aim of these anatomical studies was to demonstrate the vulnerability of these cerebral veins to rupture by mechanical stunning methods and to examine the potential of the vertebral veins to transport brain tissue emboli throughout the carcass. An involved description of the anatomy of the venous systems draining the head was not undertaken, since such detailed anatomical accounts of the cerebral sinuses and vertebral plexuses of cattle and sheep are already available (Smuts, 1977; Rauhut, 1967).

The prepared casts were successful in demonstrating the cerebral sinuses that may be traumatised by the passage of the captive bolt, and in addition, demonstrated the emissary veins that connect these sinuses to the venous systems draining the head. Furthermore, the casts provided information on the relative size of vessels within the cranium that reflects on the size of brain tissue fragments that might enter such a ruptured sinus. The vertebral plexus was shown to be a pair of vessels of significant proportions in both cattle and sheep specimens that might carry emboli throughout the carcass.

Consideration of the measured diameter of vessels of the calf venous cast suggests that emboli of up to 7-mm in diameter might enter the dorsal sagittal sinus while brain tissue fragments up to approximately 9-mm in diameter might enter the ventral sinuses. These estimates based upon measurement of vessels represented by the resin cast, are likely to be moderate exaggerations of the size of emboli that might enter a ruptured sinus. The thin walled veins may have been dilated by the pressure of resin injected under pressure through the system during the preparation of the casts. In addition, the pressure changes that occur within the cranium in the period immediately after stunning may cause very significant deviations in the size of the cerebral venous sinuses demonstrated by the cast models.

The cast of the adult bovine cerebral veins demonstrates an extensive network of voluminous vessels on the ventral aspect of the brain. The presence of these sinuses correlates well with previous observations of extensive haemorrhage on the ventral surface of the brain after stunning as a result of vessel rupture by the passage of the bolt

(Farag, 2002). It seems likely, given the large size of these ventrally situated vessels in relation to the more modest proportions of the dorsal sagittal sinus, that the ventral vessels are more likely to pick up and transport brain tissue fragments displaced by the passage of the bolt after stunning (Figure 3.18). The prepared cast also demonstrates a clear and direct connection between the basilar veins (ventral veins of the cerebral cavity) and the vertebral plexus of the spine. This communication must increase the likelihood of transport of brain tissue fragments through the spinal system of veins from the head after stunning. Examination of the cast model suggests that dissemination of brain tissue emboli following the use of CBG stunning may indeed occur by routes other than the jugular return.

Demonstration of a complete cast of the vertebral venous plexus and the cerebral sinuses is difficult, due in part, to in-complete perfusion of the venous system with resin. A second confounding factor relates to the fragility of the casts obtained after the maceration process is complete. The presence of blood clots in some veins of the head and neck specimen may have prevented full perfusion of the vertebral venous system with resin. In future studies, flushing of the system with saline and streptokinase prior to resin injection as described in other studies (Groen et al., 1997) may result in more complete casts. The delicate nature of the casts resulted in breakage and loss of delicate structures during and after the preparation process. The continuous monitoring and recording of the casts by photography through-out the maceration process may have helped to mitigate this problem and allowed reconstruction of damaged areas of the cast after the maceration process was complete. The maceration process of cast production might be improved by the use of maggot digestion of soft tissues following injection and drying of the resin (Smuts, 1977). Following removal of soft tissues by the use of maggots, the remaining bony structures could be removed by use of concentrated alkaline solutions. This modification of the cast preparation process might reduce damage of the more delicate vessels of the cast.

In addition to the use of resin injection and maceration of soft tissues, an adult ovine head and neck specimen was preserved in formalin and dissected to allow visualisation of the vertebral veins. Further dissection of similar specimens was not attempted due to limitations in the method highlighted by this trial.

Dissection of the cervical section of the specimen allowed visualisation and measurement of the vertebral vein (Figure 3.21). The diameter of vessels obtained by this method of dissection may be more accurate than measurements made from resin casts. Transverse sections made through the vertebral column revealed the relation between the vessels of the spine and the surrounding soft and bony tissues. The vessels of the vertebral column were observed to be of significant dimensions and lacking in intra-luminal valves, a feature that should allow blood and perhaps also brain tissue emboli, to flow in either direction.

Dissection of the vertebral venous system may be enhanced in future studies by the combined use of resin injection of venous structures and conventional dissection methods. This modified technique of dissection (Van der Kuip, Hoogland & Groen, 1999) should permit easier identification and isolation of venous structures.

These anatomical studies were successful in demonstrating the potential of the vertebral veins to carry brain tissue emboli from the head. Further investigation of the anatomical features and functional roles of the spinal venous systems may allow an improved estimation of the risk of emboli dissemination by this route.

## 3.3 RADIOGRAPHIC STUDY OF VENOUS CEREBRAL DRAINAGE

### 3.3.1 Introduction

In this experiment the functional characteristics of the cerebral venous system are examined. The potential of the vertebral system of veins to carry brain tissue fragments from the head after stunning has not previously been investigated. The effects of postural changes on the haemodynamics of cerebral venous outflow that might occur during stunning are investigated using routine radiographic methods on a small number of anaesthetised sheep.

### 3.3.2 Aims

- To investigate the effects of animal orientation at stunning and slaughter upon uptake and transport of brain tissue emboli by the venous circulation after application of captive bolt gun stunning in sheep.

### 3.3.3 Methodology

#### 3.3.3.1 Injection routes of contrast media

##### 3.3.3.1.1 *Intra-cranial injection via a pre-drilled hole*

A 20-ml volume of contrast media (Omnipaque, Nycomed, UK) was injected through a hole in the cranium into the estimated area of the dorsal sagittal sinus. A series of four radiographs were then taken with the animal in dorsal ventral recumbency.

##### *3.3.3.1.2 Intra-cranial injection via a bolt-hole*

Following induction of anaesthesia the animal was placed in sternal recumbency on the table. The animal was then stunned by the use of a cartridge-activated CBG used with a 1.25-grain cartridge (Cox MK 9, Accles & Shelvoke, UK). A 20-ml volume of contrast

media was then injected as a bolus through the bolt- hole. A series of two radiographs were then taken.

#### *3.3.3.1.3 Bilateral angularis oculi injection*

Following induction of anaesthesia, the animal was placed in sternal recumbency on the table and 10-ml of contrast media was injected into each angularis oculi vein. A radiograph was taken with 3-ml of media remaining in each syringe. A second radiograph was taken 30 seconds later, following injection of the remaining contrast media. The animal was inclined at approximately 40-degrees to the horizontal in a head down position by tilting the table and the injections of contrast media and radiographs were repeated.

#### *3.3.3.1.4 Dorsal sagittal sinus injection*

A hole was drilled on the mid-line of the cranium over the expected position of the vessel. Although too small to visualise the dorsal sagittal sinus this procedure did cause significant bleeding which suggested perforation of the sinus by the drill. A plastic cannula was then introduced into the hole and 10-ml of contrast media was injected by way of the cannula after confirming placement in the vein by first withdrawing blood. Two radiographs were taken immediately of the head and neck.

#### *3.3.3.1.5 One sided angularis oculi injection*

With the sheep in dorsal ventral recumbency, 10-ml of Omnipaque contrast media was injected rapidly into the angularis oculi vein and two successive dorsal ventral radiographs of the head and neck was taken. A tape around the neck was temporarily used to compress the external jugular veins during the injection of contrast solution.

While still heavily anaesthetised the animal was hoisted in a specially constructed frame to assume a head down position. The contrast media injections and x-ray photographs were repeated.

#### 3.3.3.1.6 *Bilateral angularis oculi injection*

With the animals in dorsal ventral recumbency 10-ml of Omnipaque contrast media was injected rapidly into both veins and two successive dorsal ventral view x-rays of the head and neck were taken. A tape around the neck was temporarily used to compress the external jugular veins during the injection of contrast solution.

While still heavily anaesthetised the animal was hoisted in a specially constructed frame to assume a head down position. The contrast media injections and radiographs were repeated.

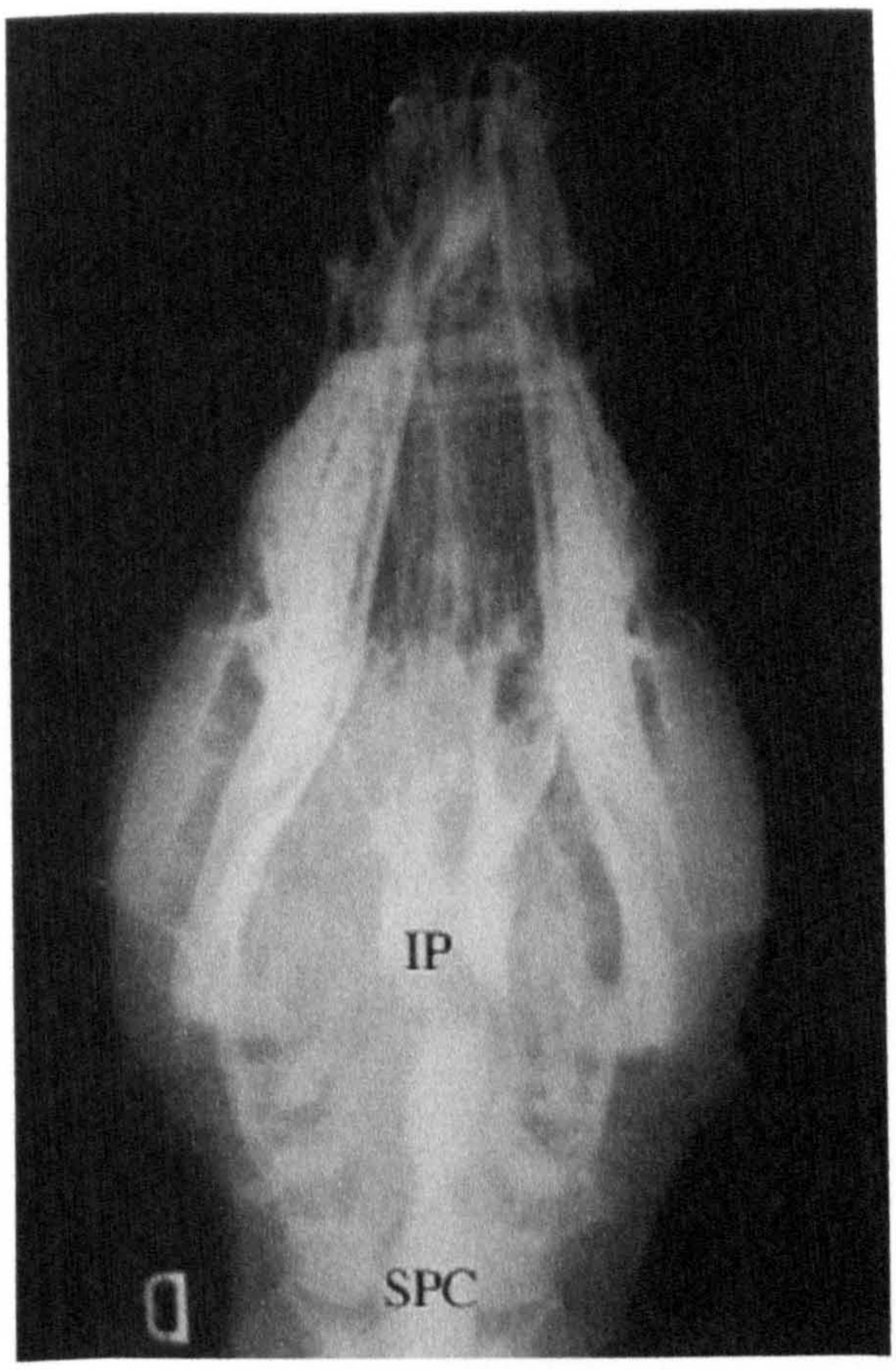
### 3.3.4 Results

#### 3.3.4.1 Intra-cranial injection via a pre-drilled hole

The substance entered the epidural space and drained down the cervical spine. No vessels of the vertebral venous system are visible suggesting that all the media was present in the epidural space.

Figure 3.22 Dorsoventral radiograph of the head following injection of contrast media through a hole drilled in the cranium.

*IP=injection point, SPC=spinal cord*

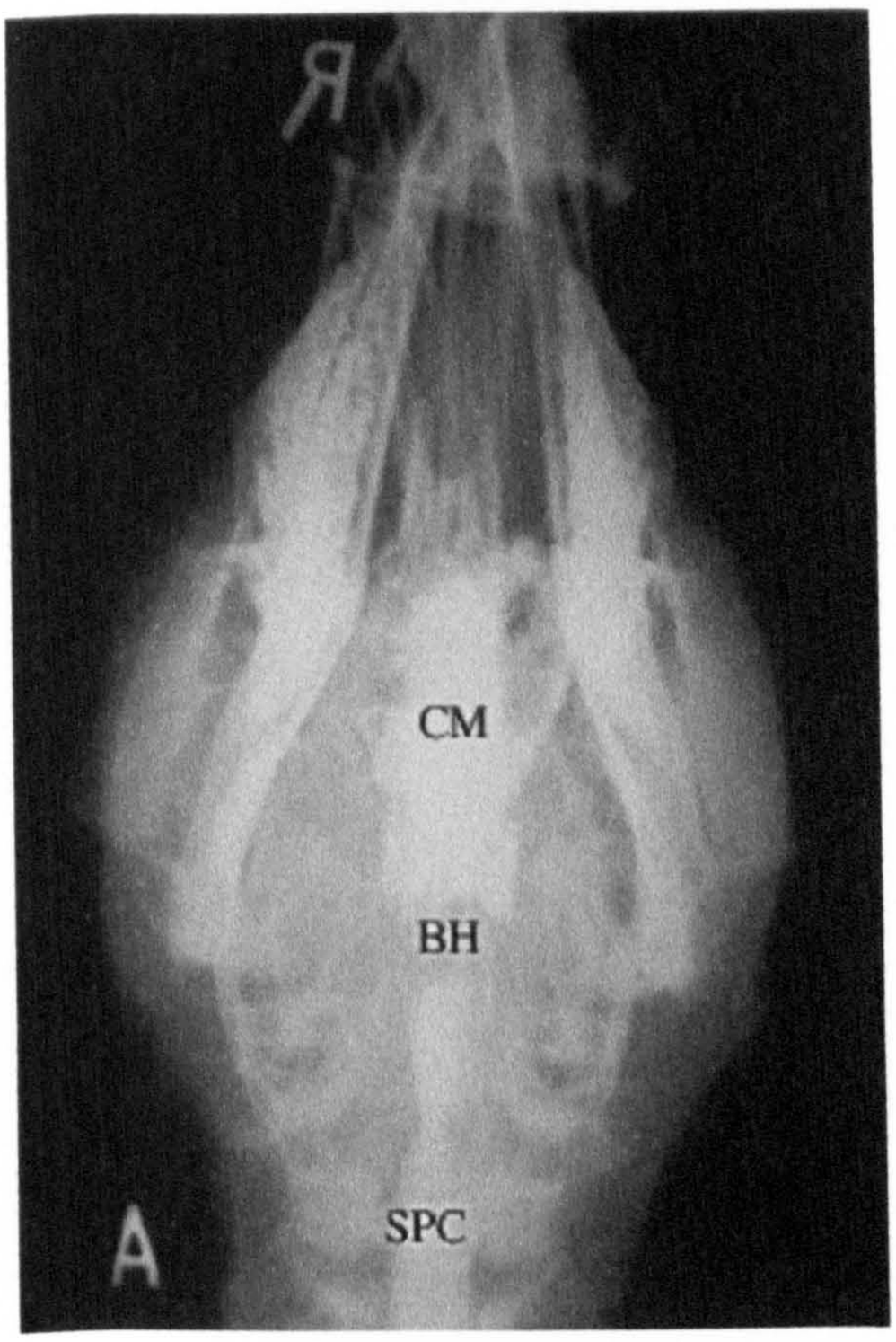



### 3.3.4.2 Intra-cranial injection via a bolt-hole

The substance entered the epidural space and drained down the cervical vertebrae within the epidural space. No vessels are visible suggesting that all the media was present in the epidural space.

Figure 3.23 Dorsoventral radiograph of the head following injection of contrast media through a bolt-hole in the cranium created by use of a penetrating captive bolt gun

*BH=bolt-bole, CM=contrast media, SPC=spinal cord*



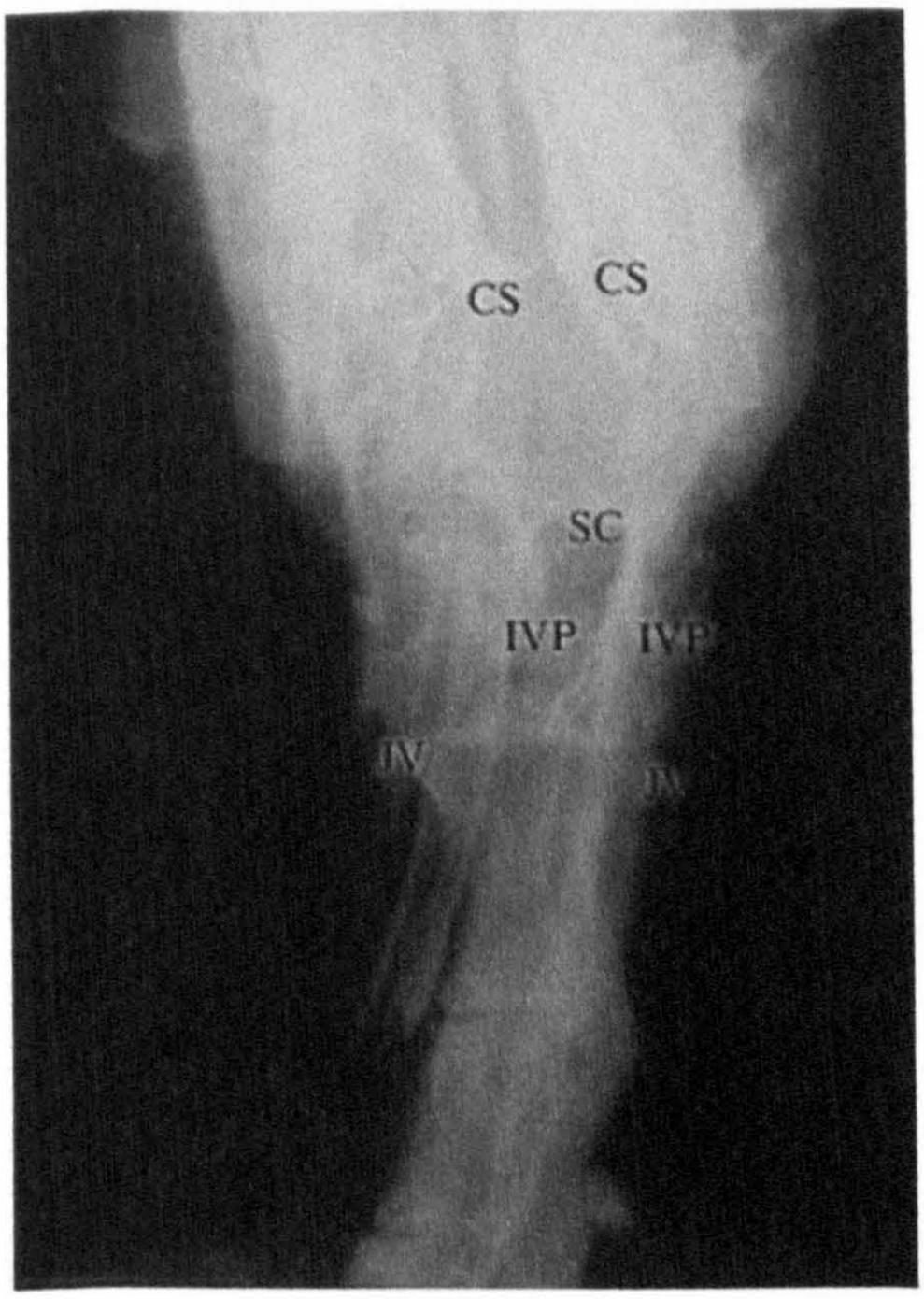

### 3.3.4.3 Bilateral angularis oculi injection

#### *3.3.4.3.1* ***Prone***

The cerebral sinuses are visible as well as both jugular veins. A small volume of contrast media has refluxed into the internal vertebral plexus although the majority of the contrast medium drains from the head by way of the jugular veins.

Figure 3.24 Dorsoventral radiograph of the head and neck following injection of contrast media into both angularis oculi veins

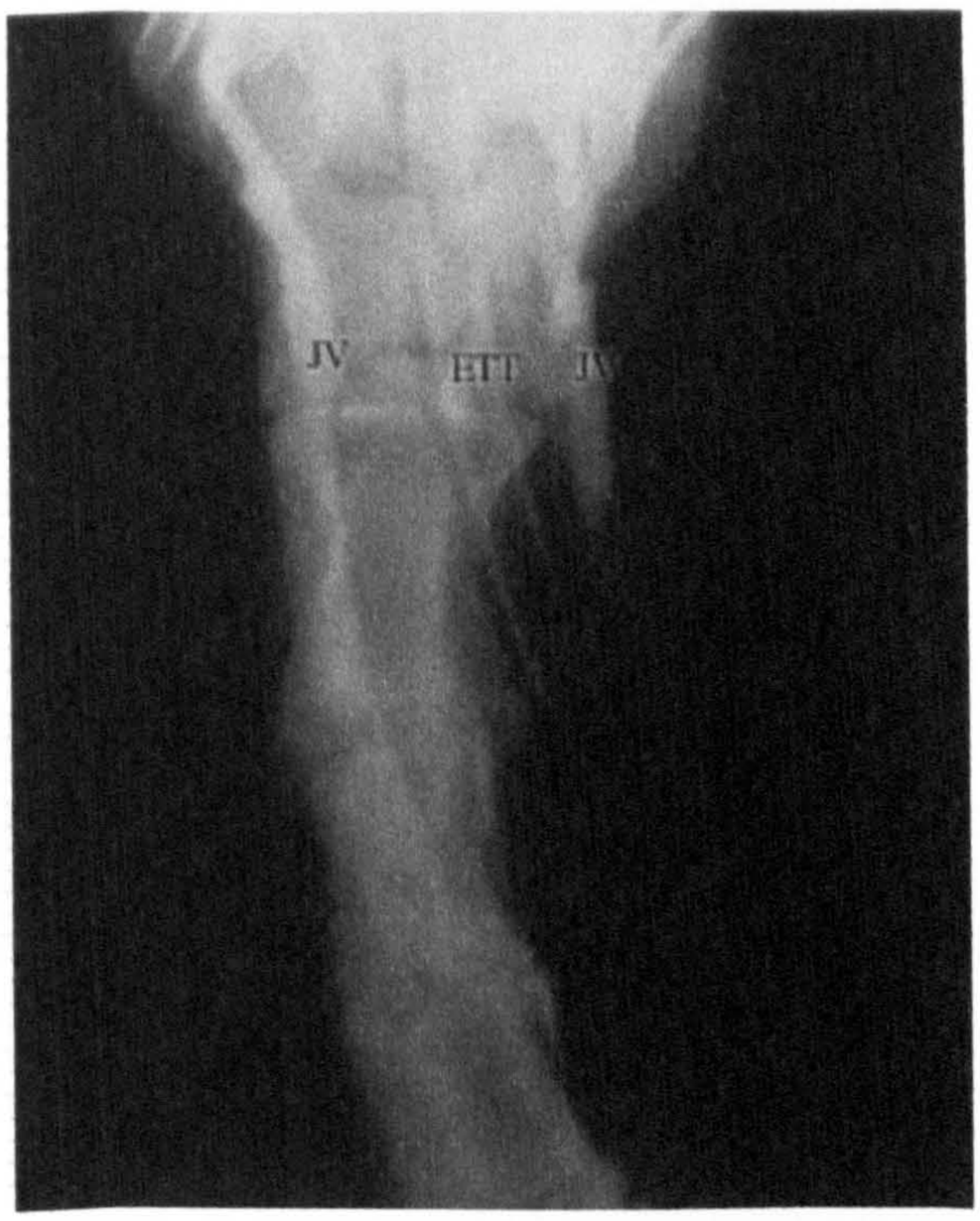
*CS=cavernous sinus; IVP=internal vertebral plexus; JV=jugular vein; SC= sinuum confluens*




### *3.3.4.3.2 Inclined at 40°to the horizontal*

Both jugular veins appear distended with contrast media while the vertebral plexus is not visible.

Figure 3.25 Dorsoventral radiograph of the skull following injection of contrast media into both angularis oculi veins with the animal tilted head-down at 40 degrees to the horizontal. *ETT=endotracheal tube; JV=jugular vein*

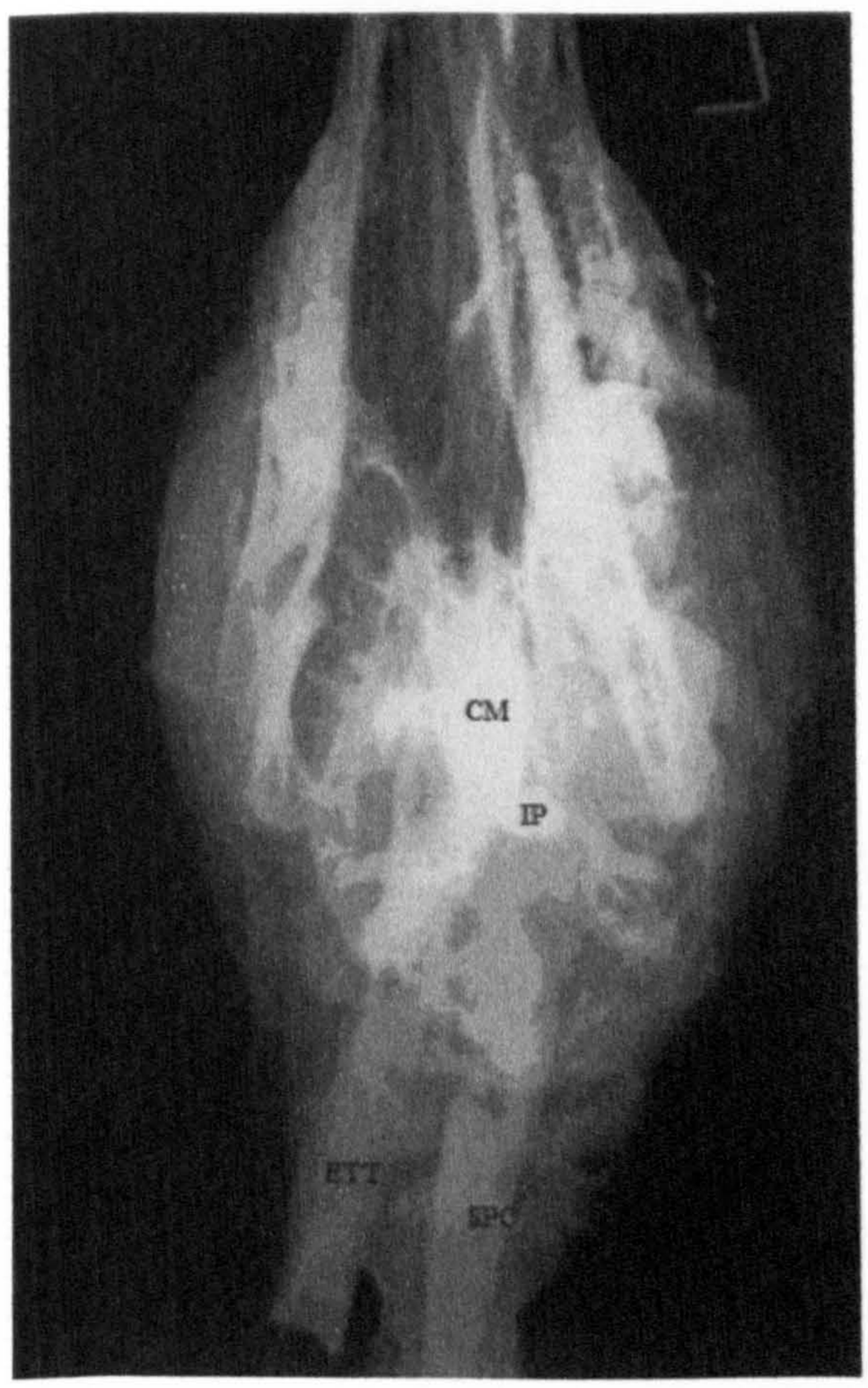



### 3.3.4.4 Dorsal sagittal sinus injection

Contrast media is faintly visible along the midline of the cranium but no veins are visible either within the cranium or along the spine.

Figure 3.26 Dorsoventral radiograph of the skull following unsuccessful attempts to cannulate the dorsal sagittal sinus and inject the contrast media by this route

*CM=contrast media; ETT=endotracheal tube; IP=injection point; SPC=spinal cord*



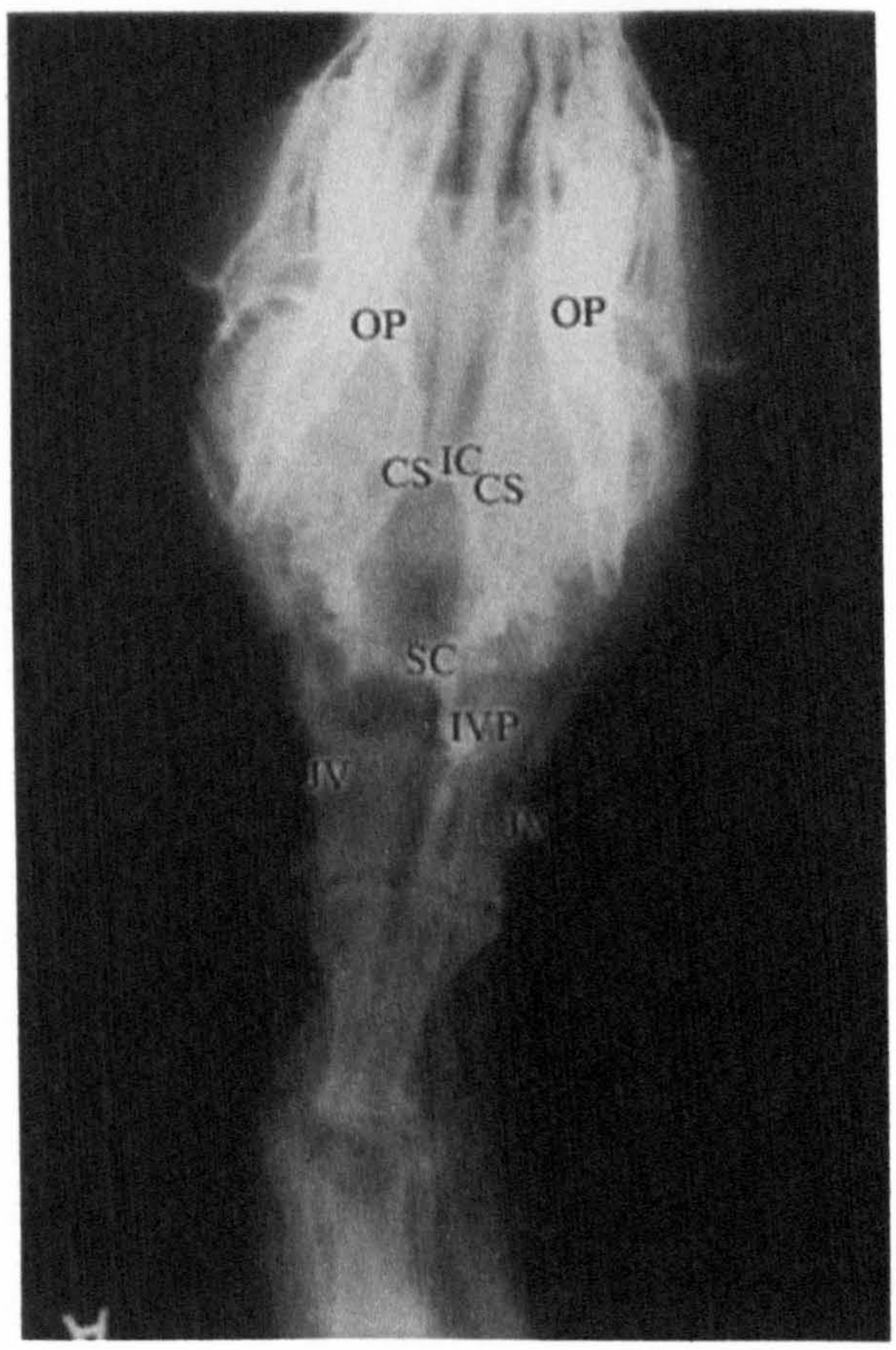
### 3.3.4.5 One sided angularis oculi injection

#### 3.3.4.5.1 *Prone*

The cerebral sinuses are visible bilaterally while the internal vertebral plexus and jugular vein are visible only on the right side.

Figure 3.27 Dorsoventral radiograph taken following injection of contrast media into the right angularis vein with the animal resting on the sternum

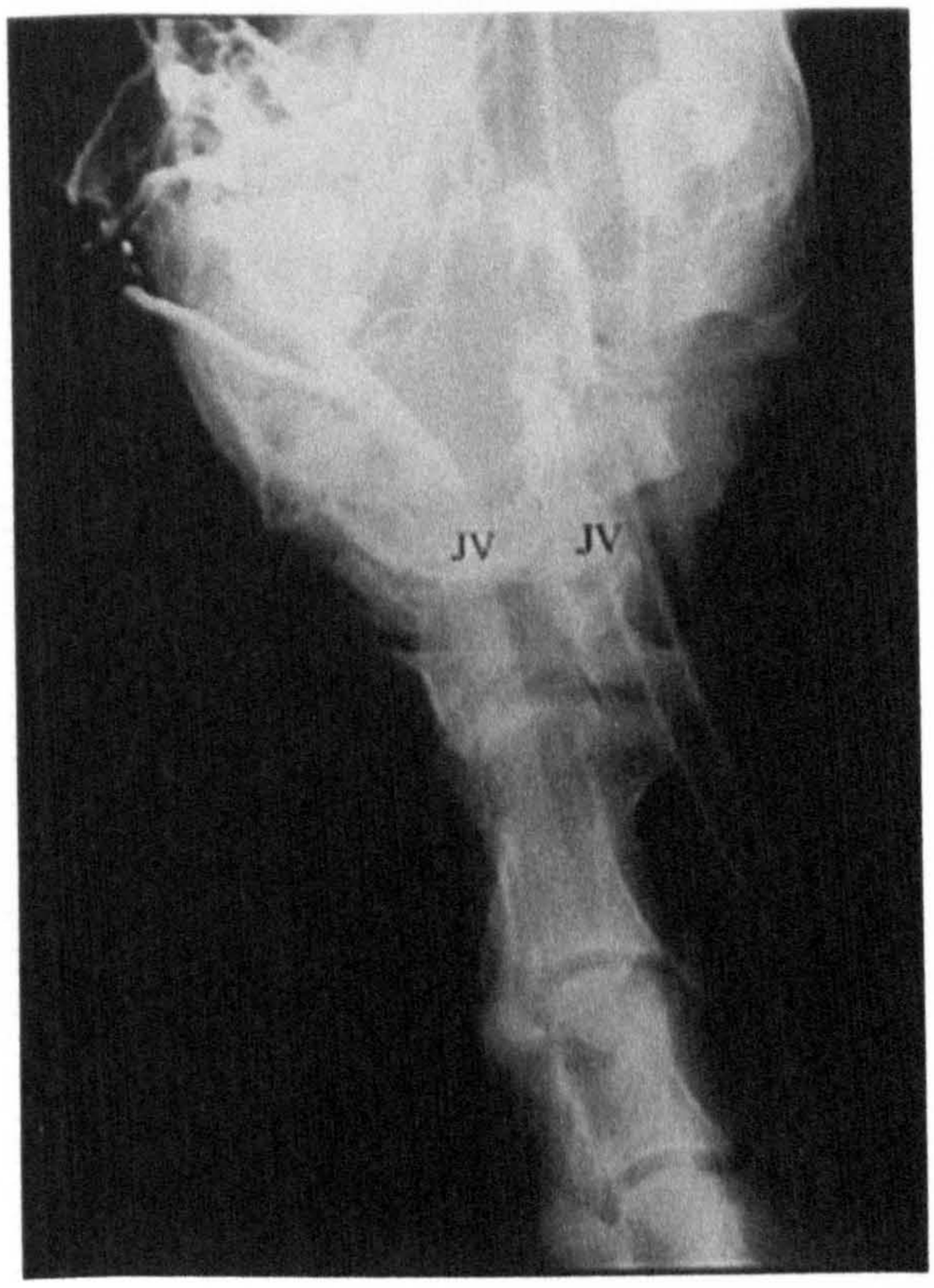
*CS=cavernous sinus; IC=intercavernous sinus; IVP= internal vertebral plexus; JV=jugular vein; OP=orbital plexus; SC=sinuum confluens*



3.3.4.5.2 ***Head down***

Both jugular veins are visible while the vertebral venous system is absent.

Figure 3.28 Dorsoventral radiograph taken following injection of contrast media into the right angularis vein with animal inclined in a head-down position
*JV=jugular vein*

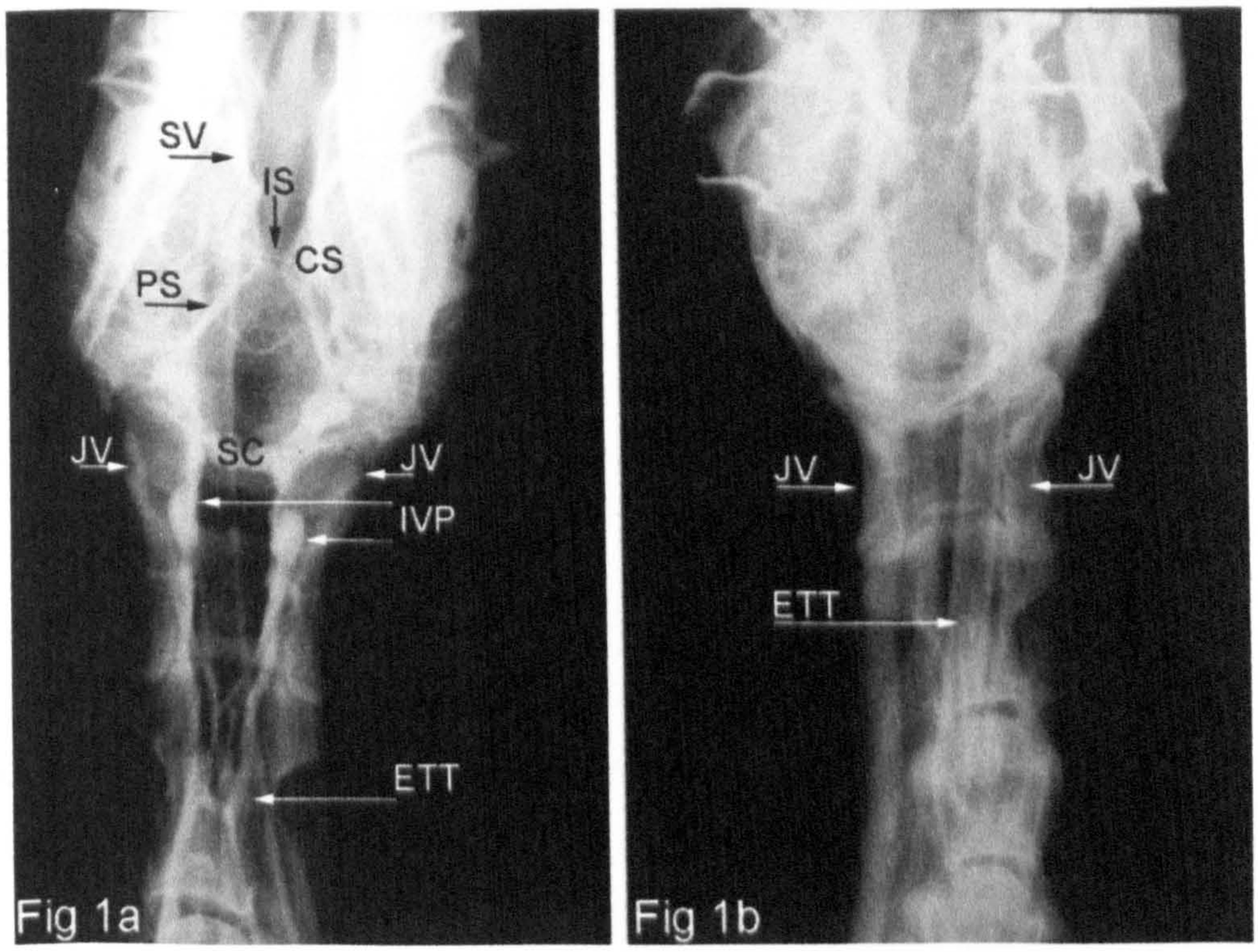



**3.3.4.6 Bilateral angularis oculi injection in a prone position (Figure 1A) and secondly in a head down position**

In the prone position the vertebral plexus is uniformly visible while the jugular veins are also visible although appears more faint in comparison. In the head down position the jugular veins are distended while the vertebral plexus is not visible.

Figure 3.29 Dorsoventral radiograph taken following injection of contrast media into both angularis oculi veins (Prone (1a) and inclined (1b))

*CS=cavernous sinus; ETT=endotracheal tube; IS= intercavernous sinus; IVP= internal vertebral plexus; JV=jugular vein; PS= petrosal sinus; SV=sphenopalatine vein*




### 3.3.5 Discussion

The vertebral venous plexus has been extensively investigated in human and animal studies using various investigative techniques including the use of radiography and ultrasound (Dilenge & Perey, 1973; Valdueza et al., 2000). Radiography using a commonly available contrast media provides adequate radiographic contrast to enable visualisation of the cerebral sinuses and the spinal veins against the background of the skull and vertebrae. Of the techniques available, radiography is particularly useful for investigations of blood flow, since the contrast media and blood are miscible and of similar viscosity. Furthermore the method requires no special equipment other than a standard x-ray unit and gives consistent results with few technical complications.

The vertebral venous plexus has previously been implicated in the transport of bacterial and cancer cells between the head and other organs of the body (Batson, 1940), other

studies suggest that the plexus may be a source for entry of air embolism to the brain and heart during surgery to the spine or skull (Epstein et al., 1970). It seems reasonable to assume that the system might also transport brain tissue fragments from the head after the use of captive bolt gun stunning.

The vertebral venous plexus deserves particular attention in relation to the dissemination of brain tissue emboli since the system is known to communicate directly with organs of the body providing a potential by-pass of blood flow around the pulmonary filter. This may mean that brain tissue fragments could lodge in organs of the carcass without passage through the lungs which filter blood returning from the head in the jugular veins.

The effects of posture on venous outflow from the head have been demonstrated in humans and in a variety of animal species (Valdueza et al., 2000; Epstein et al., 1970). By injecting a radio-opaque dye into the surgically exposed sagittal sinus of monkeys, Epstein et al., demonstrated that the vertebral system is the main drainage route for blood from the head in the standing position, while the jugular veins carry the greater volume in the supine position. The effect of a 'head-down' position on venous outflow was subsequently explored in monkeys and snakes (Dilenge & Perey, 1973; Zippel, 2001), when it was observed that the flow of blood in the vertebral system is reversed in the 'head-down'position

The findings in this experiment on anaesthetised sheep support earlier descriptions of the postural dependency of blood flow. In studies conducted on monkeys and humans, the flow of blood through the vertebral plexus in the horizontal position was minor or absent (Epstein et al., 1970; Valdueza et al., 2000), while in our studies a significant blood flow was observed. An explanation for this discrepancy may relate to the different species in which the studies were conducted. Evidence of significant blood flow through the vertebral venous plexus of recumbent sheep is significant since sheep are in a recumbent position immediately following stunning by captive bolt gun. During this time there may be potential for brain tissue fragments to drain from the head by way of the vertebral plexus.

In the perpendicular 'head-down' position and at 45°to the horizontal with the head-down, blood flow was observed only in the jugular veins. This substantiates the findings

of earlier studies that confirm the jugular veins as the only significant outflow of blood in the 'head-down' position (Zippel et al., 2001). It appears therefore that after shackling and hoisting of the animal after stunning all blood flow would occur through the jugular veins.

A variety of methods for introducing the contrast media into the cerebral sinuses were investigated. Ideally the method employed would have mimicked the entry of brain tissue fragments into a ruptured sinus after captive bolt gun stunning. Injection of the contrast material into a hole created by drilling or by the use of penetrating captive bolt gun stunning was not successful in demonstrating any uptake of contrast media into the venous circulation. If further trials were conducted using this method on larger sample numbers of sheep, uptake of contrast media may have been demonstrated.

The use of the dorsal sagittal sinus as an injection point for the contrast media was also not successful in introducing the media to the venous circulation. Considerable extravasation of the contrast medium occurred with significant volumes of media draining down the spinal cord in the epidural space. If the experiment were repeated, increasing the visualisation of the dorsal sagittal sinus by creating a larger cranial defect before attempting cannulation might increase the chances of success. Injection via the dorsal sagittal sinus has previously been described in anaesthetised dogs although the authors report, that the technique is difficult and associated with potentially serious complications (Oliver, 1969).

Injection of contrast media via the angularis oculi veins has been described in previous work (Lee and Griffiths 1972; Chawla et al., 1985). This method was successful in achieving good perfusion of the cerebral sinuses with the contrast media. Cannulation of the angularis oculi veins was aided by using a cut-down technique over the vein and by using digital pressure to straighten the vein and maintain its position under the skin. Injection of a single angularis oculi vein produced the anomaly of bilateral filling of the cerebral sinuses but only unilateral drainage of media through the vertebral plexus. The reason for this observation must relate to the arrangement of vascular communications between the angularis oculi veins and the vertebral plexus.

The prediction that the jugular veins are not the only vessels to transport blood and brain tissue emboli from the head after stunning is important for several reasons.

Firstly, estimates of the frequency of brain tissue embolism after stunning based on jugular venous samples might under-estimate the risk posed by use of these stunning methods. Secondly, transport of emboli by the vertebral venous system may cause contamination of unforeseen tissues of the carcass. Finally, control measures based on limiting carcass contamination via jugular venous drainage may not be adequate for preventing contamination of the carcass by alternative drainage routes.

The results of this study have practical implications for the stunning and slaughter of livestock and the potential for carcass contamination with brain tissue material. In abattoirs with longer stunning to sticking intervals in which the animal is left in lateral recumbency on the ground before shackling, there may be an increased risk for dissemination of brain tissue emboli by the vertebral system of veins as compared to that of abattoirs that shackle and hoist relatively rapidly.

## 3.4 SEPARATION AND QUANTIFICATION OF BRAIN TISSUE EMBOLI BY FILTRATION

### 3.4.1 Introduction

In previous studies, stunning livestock by the use of captive bolt guns has been shown to sometimes cause embolism of brain tissue fragments into the jugular vein blood (Anil et al., 1999; 2001). Methods have been developed for detection of brain tissue fragments in minute aliquots of the blood samples collected. Although these methods are sensitive, the non-homogeneous nature of brain tissue fragments in blood does not allow measurement of the amount of brain tissue entering the venous circulation after stunning. The amount of brain tissue that may comprise an embolus is clearly a critical determinant of any risk assessment of CBG stunning and possible prion protein contamination of the carcass by way of infected brain tissue fragments carried in the blood. A method is needed where by the entire volume of blood that may potentially carry brain tissue fragments, is processed to allow separation of all suspended brain tissue fragments and blood. The isolated brain tissue may then be weighed to give a more accurate estimation of the total brain tissue mass that enters the circulation after use of captive bolt gun stunning methods.

Initial bench studies on the potential of leukocyte filters to remove known amounts of brain tissue fragments from blood samples indicated that much of the brain tissue was retained in the pre-filter part of the filter system (Mr. A. Shand, Department of Neurosciences, Frenchay hospital, Bristol, UK-personal communication). Any material within the leukocyte filter itself was very difficult to recover although back flushing with buffers was attempted. Based on these studies, we have attempted to remove fragments of brain tissue that may be present in jugular blood after captive bolt gun stunning of sheep, by filtration of blood using the pre-filter component of the leukocyte filtration system.

### 3.4.2 Aims

- To investigate the use of a leukocyte filtration system for separation from blood of brain tissue emboli created by the application of captive bolt gun stunning in sheep.
- To quantify the amount of brain tissue emboli released into the venous circulation after captive bolt gun stunning.

### 3.4.3 Methodology

Fifteen adult sheep were used in this study. The animals ranged in weight from 20 to 60-kg. Animals were anaesthetised and catheters were placed in the jugular veins of each animal. Eight sheep were then stunned by cartridge-activated captive bolt gun and seven by pneumatically-activated captive bolt gun and jugular blood samples were taken. Blood samples were filtered and captured tissue in each pre-filter was weighed and sent for analysis using the combined techniques of microscopy and immunocytochemistry. The filtrate was analysed by ELISA for the brain protein GFAP.

### 3.4.4 Results

Visible quantities of tissue were recovered in the pre-filters from ten of the fifteen sheep sampled. Analysis of these samples using the combined techniques of microscopy and immunocytochemistry indicated that six of the ten samples contained some brain tissue suggesting that 40% of the animals sampled had brain tissue embolism after stunning (frequency of 0.4, 95% confidence interval of 0.198 to 0.643). The weights of tissue recovered from these confirmed samples ranged from 0.22-g to 2.08-g. The mean weight of brain tissue obtained from these six samples was 0.97-g (SD=0.81). Although positive samples identified by microscopy were confirmed by staining with two antibody stains, only a proportion of the tissue sample recovered in each pre-filter was brain tissue (Prof. S. Love, Department of Neuropathology, Frenchay Hospital, Bristol, UK-personal communication). The remainder may have comprised fibrin and retained blood cells. The filtrate from a single animal sample confirmed the presence of elevated

levels of GFAP from a sample in which 1.75-g of tissue was captured in the pre-filter all other filtrate samples analysed by ELISA were negative for GFAP.

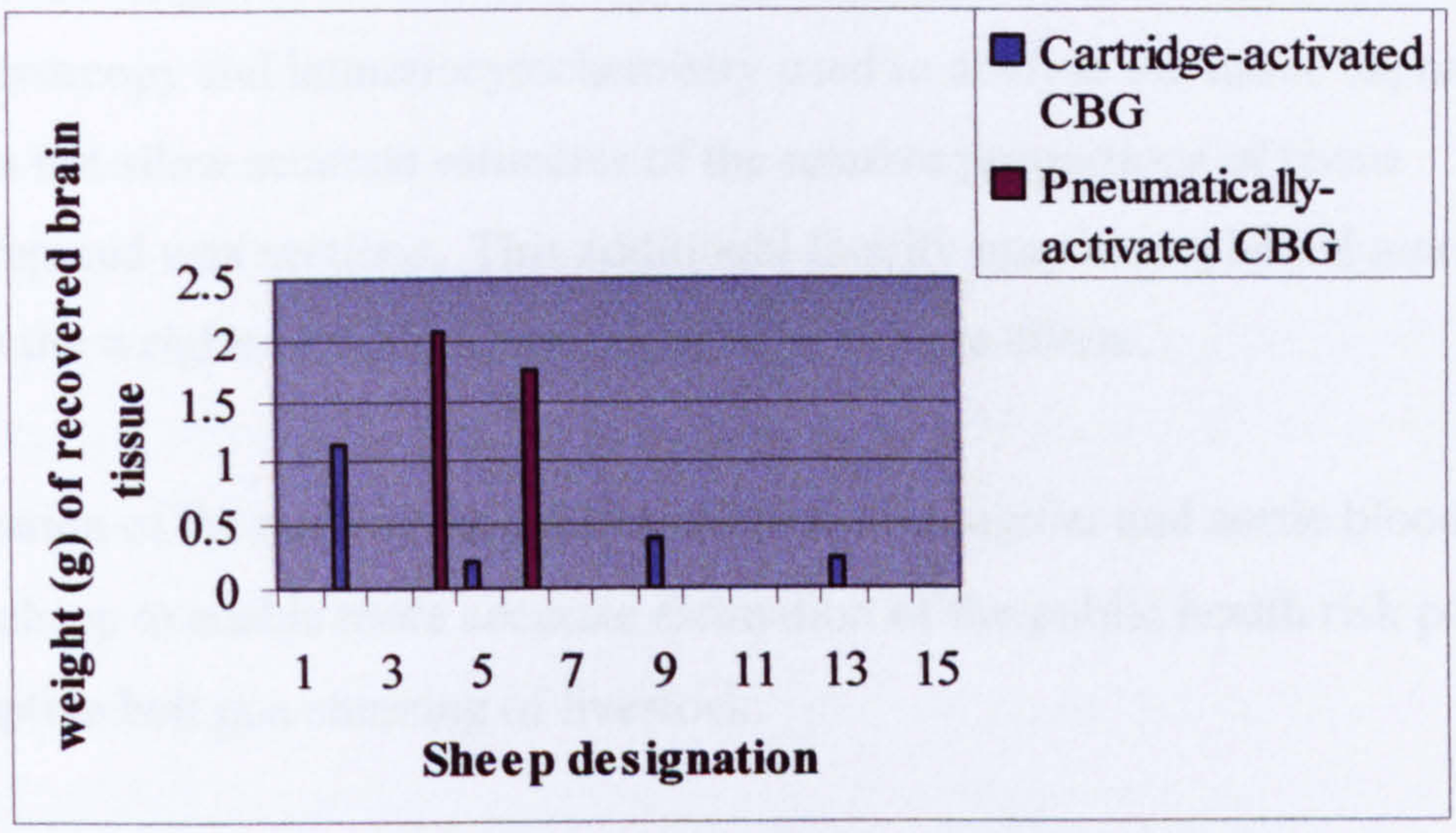
Table 3.23 Weight of brain tissue recovered from each filter following filtration of jugular blood samples from sheep after application of captive bolt gun stunning

| Animal number | Sample designation. (QS) | CBG | Filter number | Pre-filter wt. (g) | Post-filter wt. (g) | Tissue wt. (g) | Confirmed positive by immunocytochemical analysis and ELISA Yes (√)/No (x) |
|---|---|---|---|---|---|---|---|
| 1 | 2 | Cartridge | - | - | - | - | |
| 2 | 6,7,8 | Cartridge | QSF2 | 1.49 | 2.63 | 1.14 | √ |
| 3 | 10, 11,12 | Pneumatic | QSF3 | 1.50 | 1.65 | 0.15 | x |
| 4 | 14, 15, 16 | Pneumatic | QSF4<br>QSF5 | 1.63<br>1.48 | 3.00<br>2.19 | 1.37<br>0.71<br>2.08 | Clotted<br><br>√ |
| 5 | 18, 19, 20 | Cartridge | QSF6 | 1.62 | 1.84 | 0.22 | √ |
| 6 | 22, 23, 24 | Pneumatic | QSF1 | 1.51 | 3.26 | 1.75 | + GFAP ELISA<br>√ |
| 7 | 26, 27, 28 | Cartridge | QSF8 | - | - | - | |
| 8 | 30, 31, 32 | Pneumatic | QSF9 | 1.64 | 1.84 | 0.20 | x |
| 9 | 34, 35, 36 | Cartridge | QSF10 | 1.64 | 2.04 | 0.4 | √ |
| 10 | 38, 39, 40 | Pneumatic | QSF11 | 1.49 | 1.58 | 0.09 | x |
| 11 | 41, 42, 43 | Cartridge | QSF 12 | 1.60 | 1.89 | 0.29 | x |
| 12 | 46 | Pneumatic | QSF 13 | 1.49 | 1.49 | 0.00 | - |
| 13 | 49,50,51,52 | Cartridge | QSF 14 | 1.49 | 1.83 | 0.24 | √ |
| 14 | 53,54,55,56 | Pneumatic | QSF 15 | 1.61 | 1.61 | 0.00 | - |
| 15 | 58 | Cartridge | QSF 16 | 1.63 | 1.63 | 0.00 | - |

-=no sample

√=confirmed positive by microscopy and immunocytochemistry

Figure 3.30 Weights of tissue obtained in venous blood following captive bolt gun stunning of sheep




### 3.4.5 Discussion

The results of this experiment indicated that filtration of jugular blood samples after stunning may provide a more efficient and cost effective method of separating brain tissue from blood than the previously described techniques of buffy coat isolation by centrifugation (Anil et al., 1999; Love et al., 2000). The filtration of samples offers several advantages over this previously described method. Firstly, filtration of blood samples is faster allowing processing of greater volumes of blood from a single animal and consequently increasing the chances of identifying positive samples. The technique has the additional advantage of allowing quantification of the brain tissue captured in the filter. Furthermore, the capture of brain tissue emboli in filters and preservation in formalin reduces the labour intensiveness of the analytic methods since such samples can be stored indefinitely without adverse effects in quality until further processing. This is in contrast to the buffy coat samples that must be processed within twenty-four hours of separation. The results from this pilot study indicate a frequency of brain tissue in the jugular venous return of 6/15 (40%) that is considerably higher than found in the frequency study of brain tissue embolism after stunning. This raises the possibility that the method of centrifugation may have under-estimated the frequency of embolism in that sample population. The finding of up to 2-g of tissue in the jugular blood provides the first experimental estimates of the quantity of brain tissue that may enter the venous return at stunning. It is likely that only a fraction of the tissue

collected in the filter was of CNS origin (Prof. Seth Love, Department of Neurosciences, Frenchay hospital, Bristol, UK), nevertheless, the estimate does provide an upper limit of the weight of brain tissue in the captured blood. Unfortunately, the technique of microscopy and immunocytochemistry used to analyse the tissue captured in the filter, does not allow accurate estimates of the relative proportions of tissue present in the prepared wax sections. This additional facility may have yielded more accurate data on the weights of brain tissue captured in the pre-filters.

Further investigation of the method should be conducted on jugular and aortic blood from cattle and sheep to enable more accurate estimation of the public health risk posed by the use of captive bolt gun stunning of livestock.

# 3.5 POLYMERASE CHAIN REACTION FOR DETECTION OF MALE DNA IN TISSUES OF FEMALE ORIGIN

## 3.5.1 Introduction

This series of experiments investigates the application to ovine tissue, a real-time PCR originally developed for use with bovine tissues. Secondly, this experiment attempts to use the assay to determine the extent of dissemination of brain tissue fragments within the ovine carcass after CBG stunning. Furthermore, the method offers potential for estimation of the amount of carcass contamination with brain tissue that occurs following release of brain tissue fragments into the circulation after the use of captive bolt gun stunning methods.

## 3.5.2 Aims

- To validate a real-time PCR assay previously developed for use with bovine DNA using ovine DNA.
- To use the assay to investigate contamination of the ovine carcass with brain tissue emboli following the application of captive bolt gun stunning.

## 3.5.3 Methodology

### 3.5.3.1 Amplification of male and female bovine tissue samples

Initial trials with the real-time PCR were conducted using bovine tissues supplied by Dr. C. Helps, (University of Bristol, UK) in order to gain familiarity with the technique.

### 3.5.3.2 Amplification of male and female ovine tissue samples

Although this real-time PCR was previously validated using bovine DNA, the amplification of ovine DNA using the same primers and probes has not previously been attempted. Comparison of the specific gene sequences obtained from Genbank indicated that the primer sequences in sheep and cattle were identical suggesting that the

assay should also work using ovine DNA. A difference of one base was observed in the probe sequence. The objective of this experiment was to examine the application of the real-time PCR assay to amplify ovine SRY chromosomal DNA and ovine 28S rDNA.

### 3.5.3.3 Efficiency of amplification of male ovine DNA extracted from brain tissue

The efficiency of amplification of this real-time PCR assay was determined from the slope of the regression lines generated after real-time amplification of ten-fold serial dilutions of the ovine male DNA. The computer software (Bio-rad) using the equation 100 x 10 (-1/slope) -1 calculated the percentage efficiency of amplification.

### 3.5.3.4 Dilutions of male ovine tissue in female ovine tissue

Known amounts of brain tissue from a male animal were homogenised and combined with weighed quantities of homogenised tissue from a female animal. The first dilution contained 10-mg of male brain tissue in 2000-g of female brain tissue (0.5%) (U2) while the second contained 50-mg of male brain tissue in 2000-g of female brain tissue (2.5%) (U3). Samples of these prepared dilutions along with positive and negative controls were then analysed by real-time PCR following extraction of DNA from each sample.

### 3.5.3.5 Detection of male ovine brain tissue in organs of a female ovine carcass

In this study male brain tissue suspensions were injected into the jugular vein blood of a female animal while simultaneously stunning using a penetrating captive bolt gun. After exsanguination and slaughter, specified organs were removed from the carcass processed and then analysed by real-time PCR for the presence of the marker male DNA.

### 3.5.4 Results

#### 3.5.4.1 Amplification of male and female bovine tissue samples

The Y chromosome DNA was detected at a threshold value similar to that obtained in previous trials of the assay (Dr. C.R. Helps, University of Bristol, personal communication). Both male and female DNA samples produced similar threshold values of 28S rDNA.

Table 3.24 SRY probe threshold values for bovine male DNA

| Well | $C_t$ Value | Template |
|---|---|---|
| D4 | 23.5* | SRY |
| D5 | 23.7* | SRY |
| D6 | 0.00 | Female 28S rDNA |
| D7 | 0.00 | Female 28S rDNA |
| D8 | 0.00 | Water (-control) |
| D9 | 0.00 | Water (-control) |

Figure 3.31 SRY probe threshold values of bovine DNA

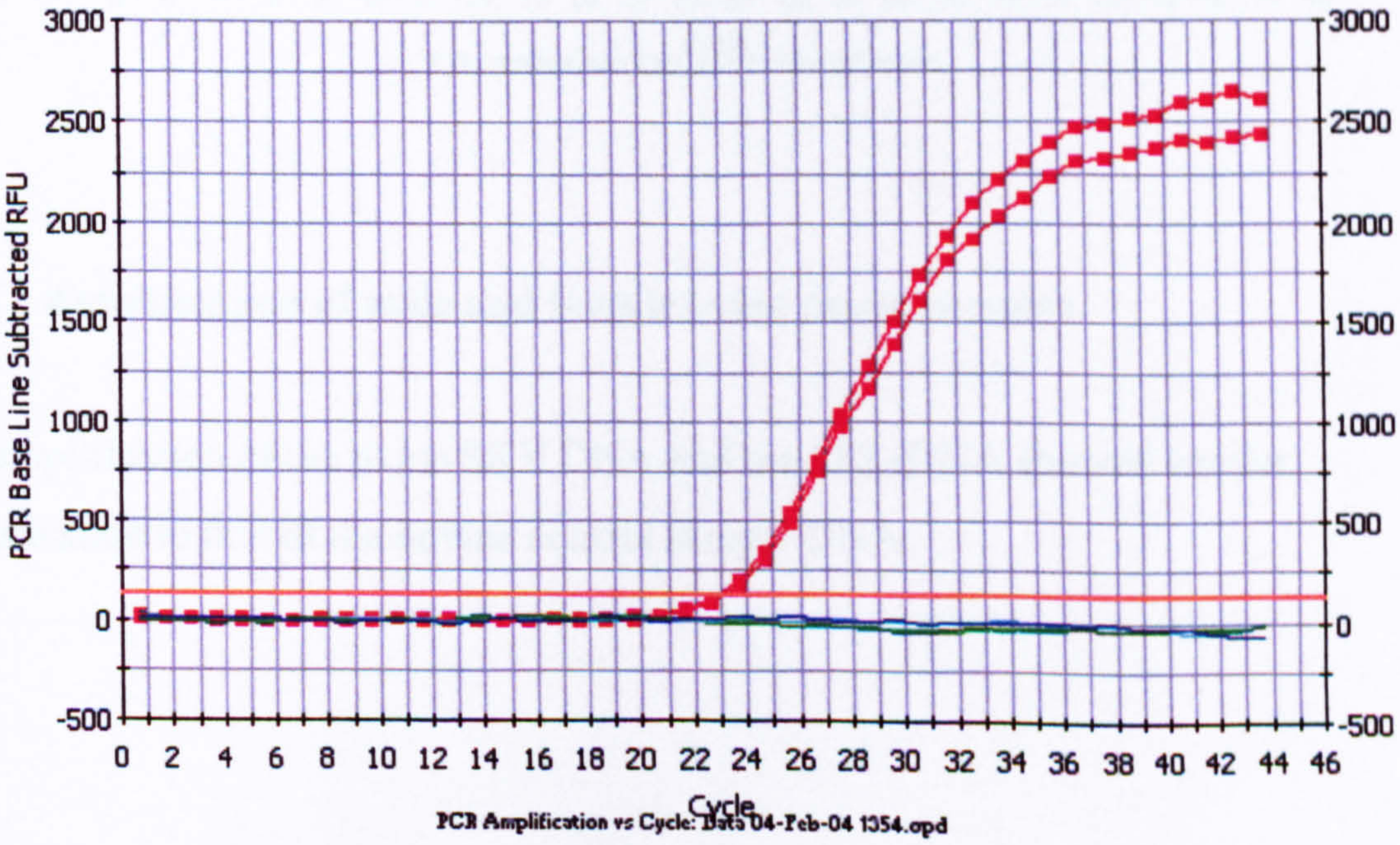
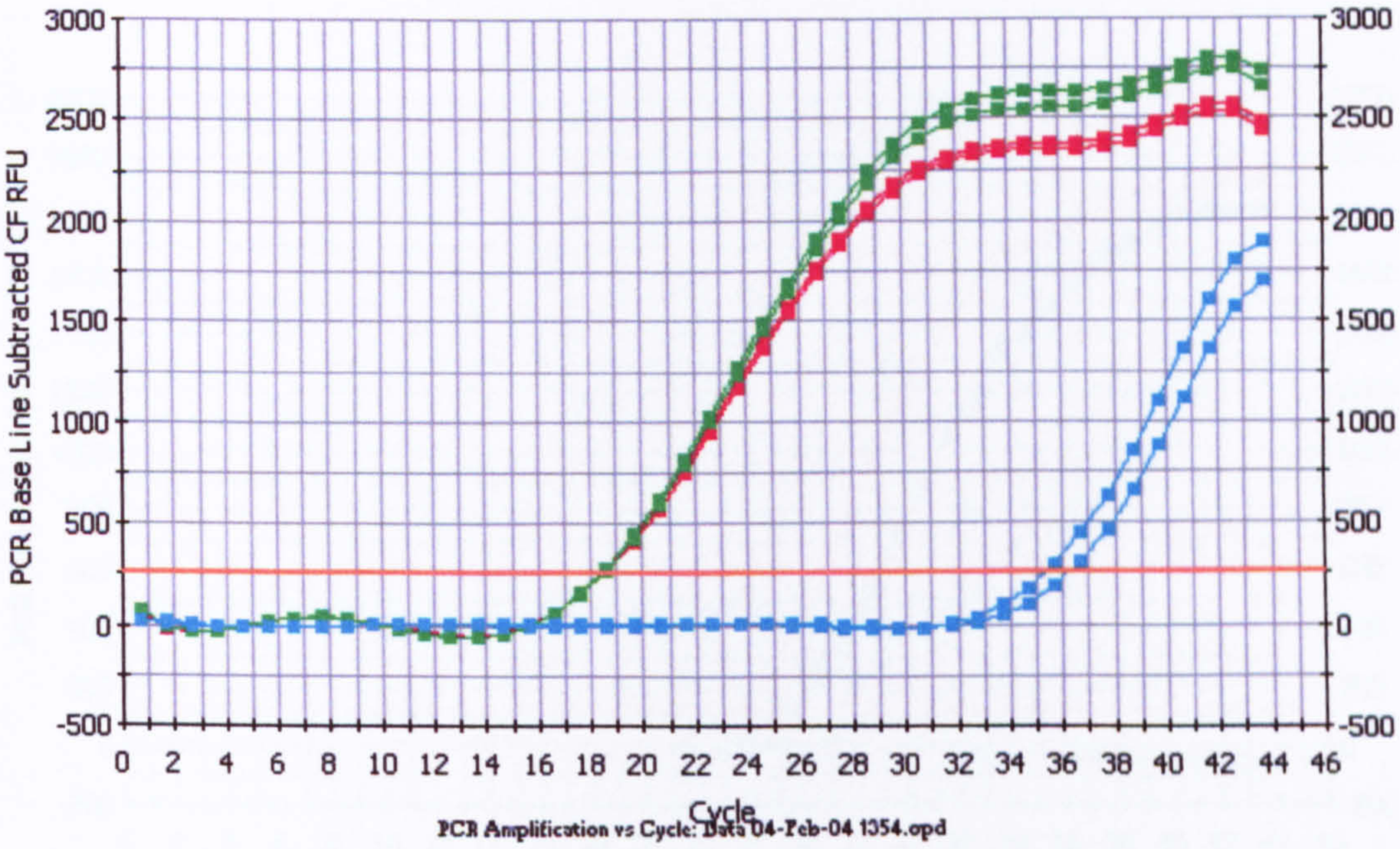


Table3.25 28S probe threshold values for bovine male and female DNA

| Well | Ct value (250) | Template |
|---|---|---|
| D4 | 19.0* | Male 28S rDNA |
| D5 | 19.1* | Male 28S rDNA |
| D6 | 19.0* | Female 28S rDNA |
| D7 | 18.9* | Female 28S rDNA |
| D8 | 35.6* | Water (-control) |
| D9 | 36.5* | Water (-control) |

Figure 3.32 28S probe threshold values of bovine DNA and control




### 3.5.4.2 Amplification of male and female ovine tissue samples

The amplification of the ovine SRY DNA and the 28S rDNA showed similar amplification to that of the bovine control sample DNA.

Table 3.26 SRY probe threshold values for ovine male DNA

| Well | Ct | Template |
|---|---|---|
| C3 | 25.6* | Male ovine |
| C4 | 25.4* | Male ovine |
| C5 | 0.00 | Female ovine |
| C6 | 0.00 | Female ovine |
| C7 | 0.00 | water |
| C8 | 0.00 | water |
| C9 | 23.6* | Male bovine |
| C10 | 23.6* | Male bovine |

Figure3.33 SRY probe threshold values of male ovine DNA and control

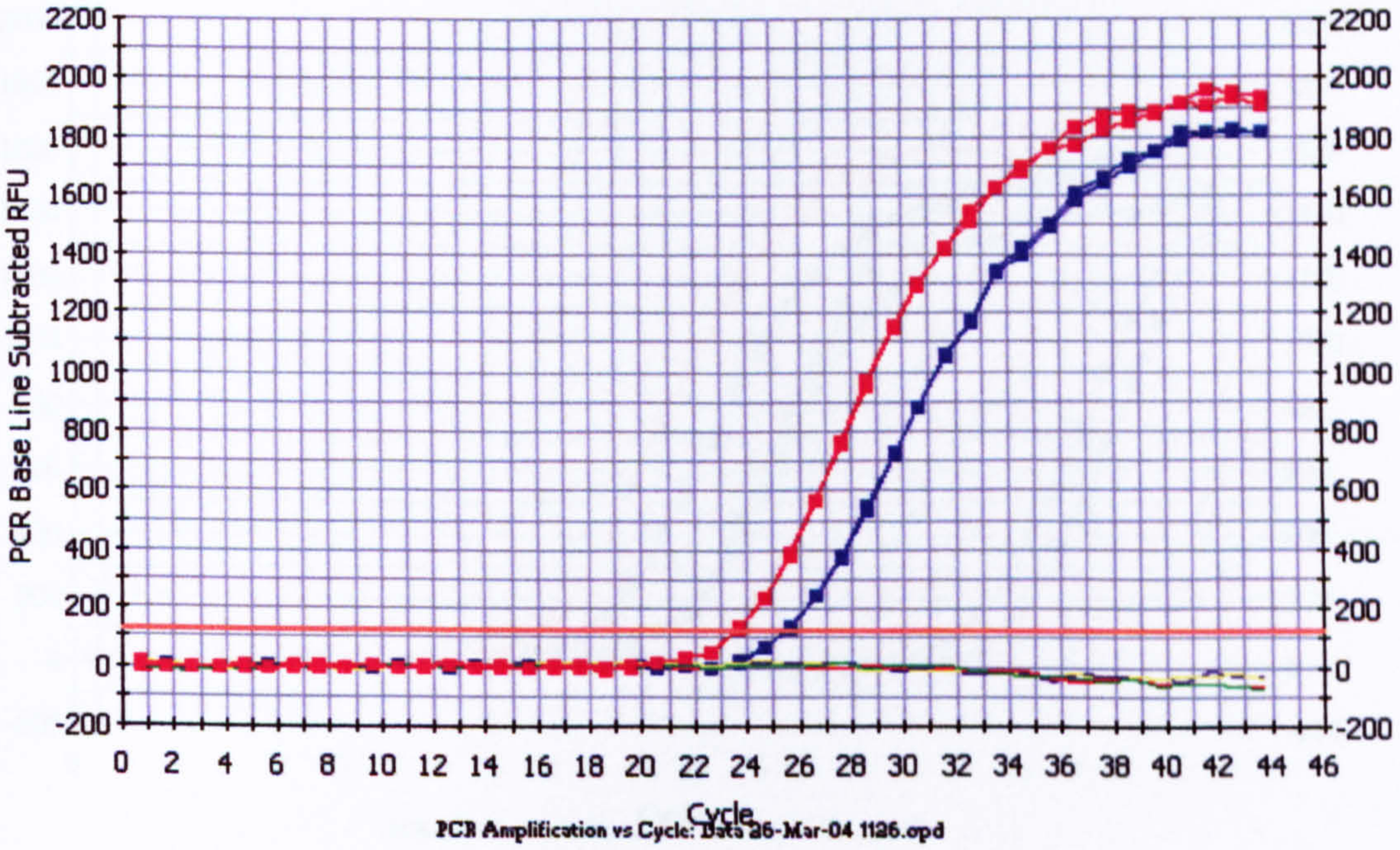
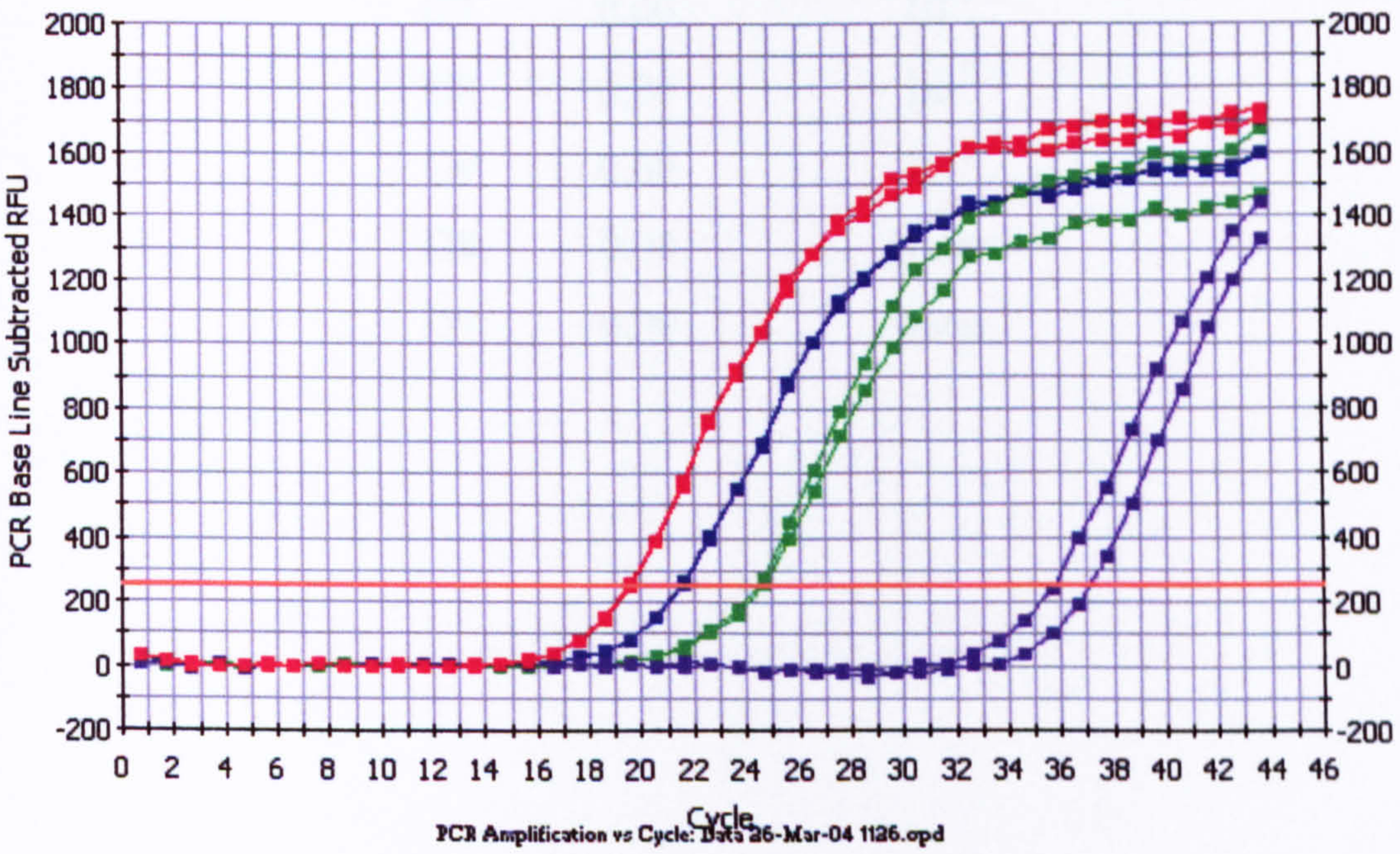


Table 3.27 28S probe threshold values for ovine male and female DNA

| Well | Ct | Template |
|---|---|---|
| C3 | 21.5* | Male ovine |
| C4 | 21.6* | Male ovine |
| C5 | 24.6* | Female ovine |
| C6 | 24.4* | Female ovine |
| C7 | 37.1* | water |
| C8 | 35.8* | water |
| C9 | 19.6* | Male bovine |
| C10 | 19.5* | Male bovine |

Figure 3.34 28S probe threshold values of ovine DNA and control samples




### 3.5.4.3 Efficiency of amplification of male ovine DNA extracted from brain tissue

Serial 10-fold dilutions of male sheep DNA using FAM-probe showed an efficiency of 103.1% with a slope of -3.251 (Figure 3.34) and demonstrated linearity over a $10^{-3}$ range of starting templates.

Serial 10-fold dilutions of male sheep DNA using the Texas-red probe showed an efficiency of 103.9% with a slope of -2.575 (Figure 3.36) and demonstrated linearity over a $10^{-4}$ range of starting templates.

The detection limit of the assay for male brain tissue was 100µg/ml [20mg (starting quantity of brain tissue)/40,000 (dilution factor)] or 0.05%.

Table 3.28 SRY probe threshold values for dilutions of ovine male DNA

| Well | Ct value | template |
|---|---|---|
| C3 | 24.4* | Stock Male DNA |
| C4 | 28.1* | $10^{-1}$ |
| C5 | 31.9* | $10^{-2}$ |
| C6 | 34.2* | $10^{-3}$ |
| C7 | 0.00 | $10^{-4}$ |
| C8 | 0.00 | $10^{-5}$ |
| C9 | 0.00 | 10-$^{6}$ |
| D8 | 0.00 | water |
| D9 | 0.00 | water |

Figure 3.35 SRY probe threshold values of dilutions of ovine DNA

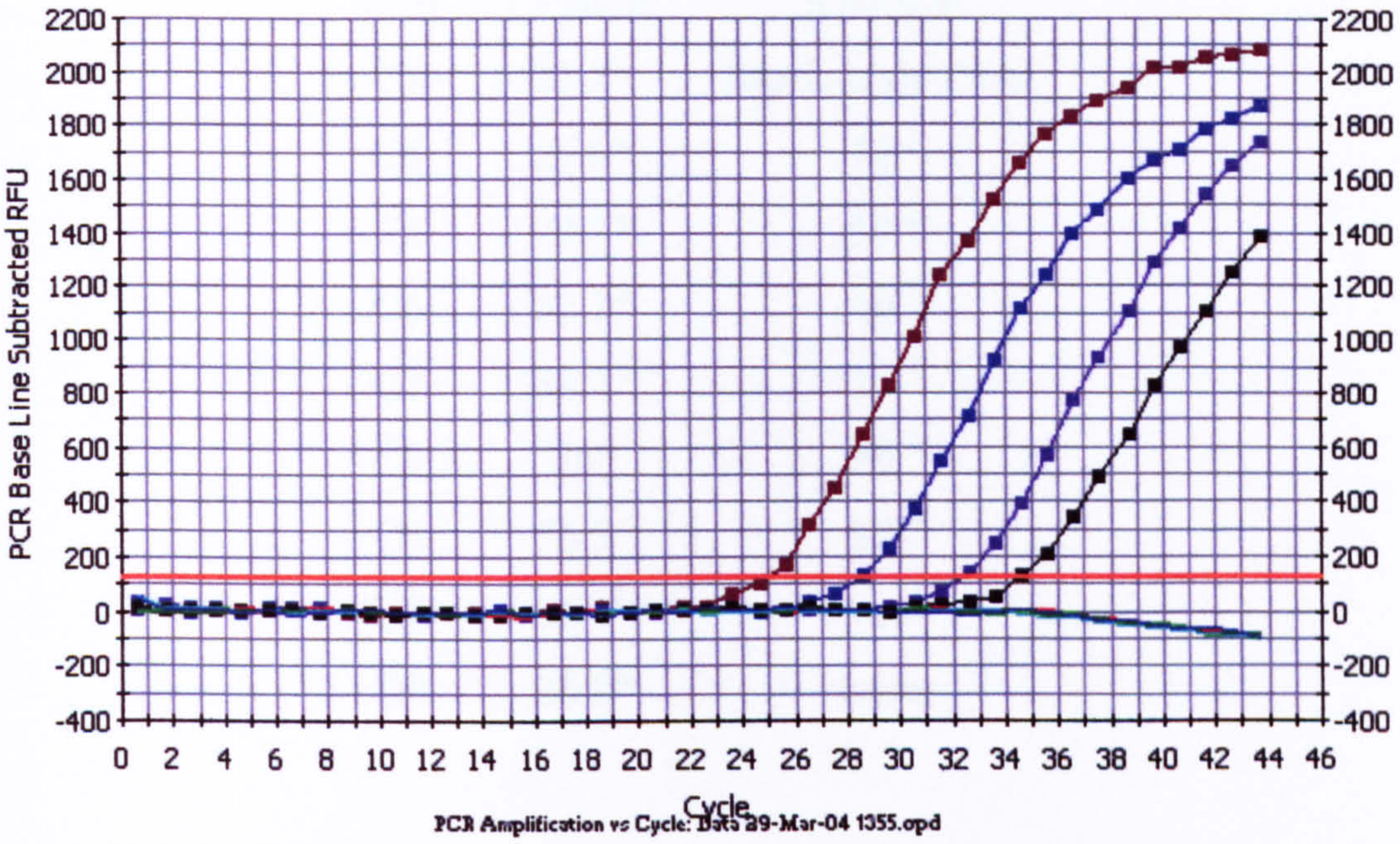



Figure 3.36 Standard curve of dilutions of ovine male DNA generated by plotting the Ct values of the SRY probe against the logarithm of the initial copy numbers

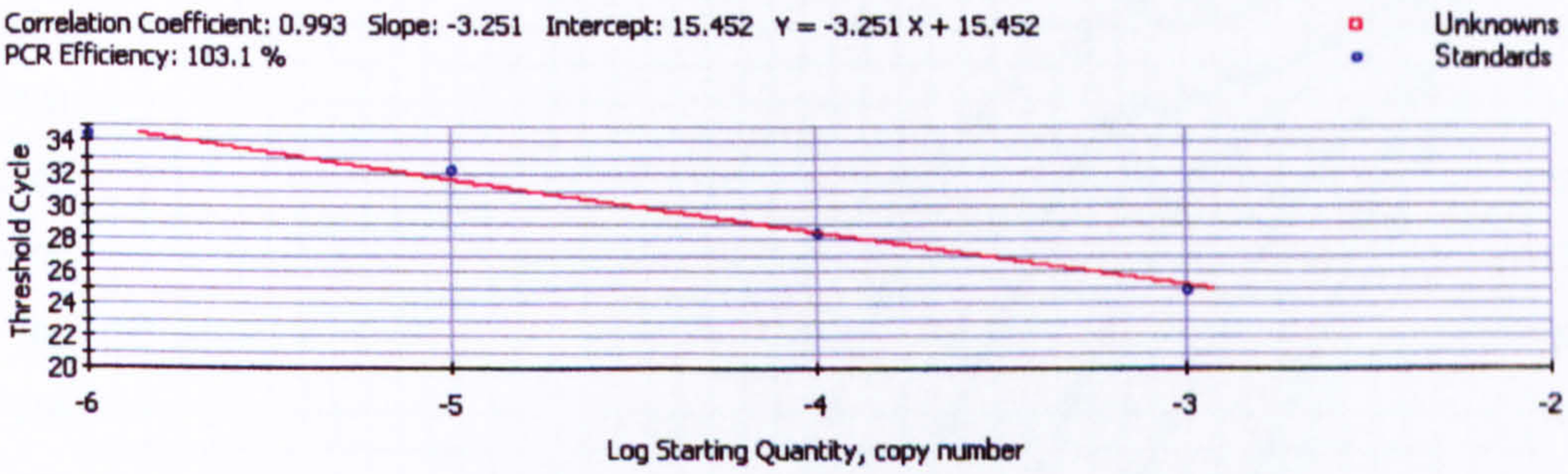


Table 3.29 28S probe threshold values for dilutions of ovine male DNA

| Well | Ct value | template |
|---|---|---|
| C3 | 21.5* | Stock Male DNA |
| C4 | 25.6* | $10^{-1}$ |
| C5 | 29.1* | $10^{-2}$ |
| C6 | 32.2* | $10^{-3}$ |
| C7 | 35.3* | $10^{-4}$ |
| C8 | 36* | $10^{-5}$ |
| C9 | 36.5* | 10-$^{6}$ |
| D8 | 36.1* | water |
| D9 | 36.8* | water |

Figure 3.37 28S probe threshold values of ovine DNA

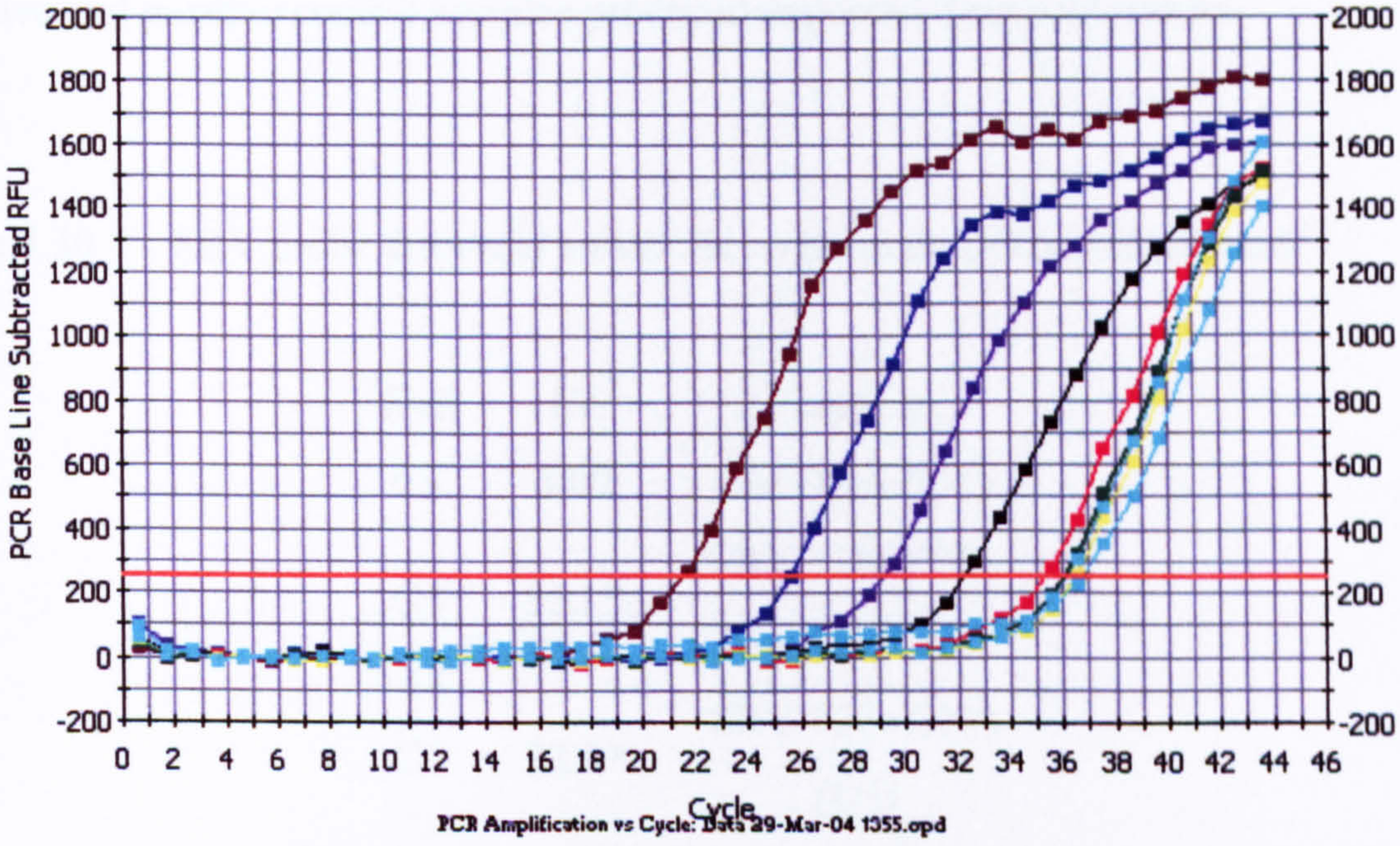
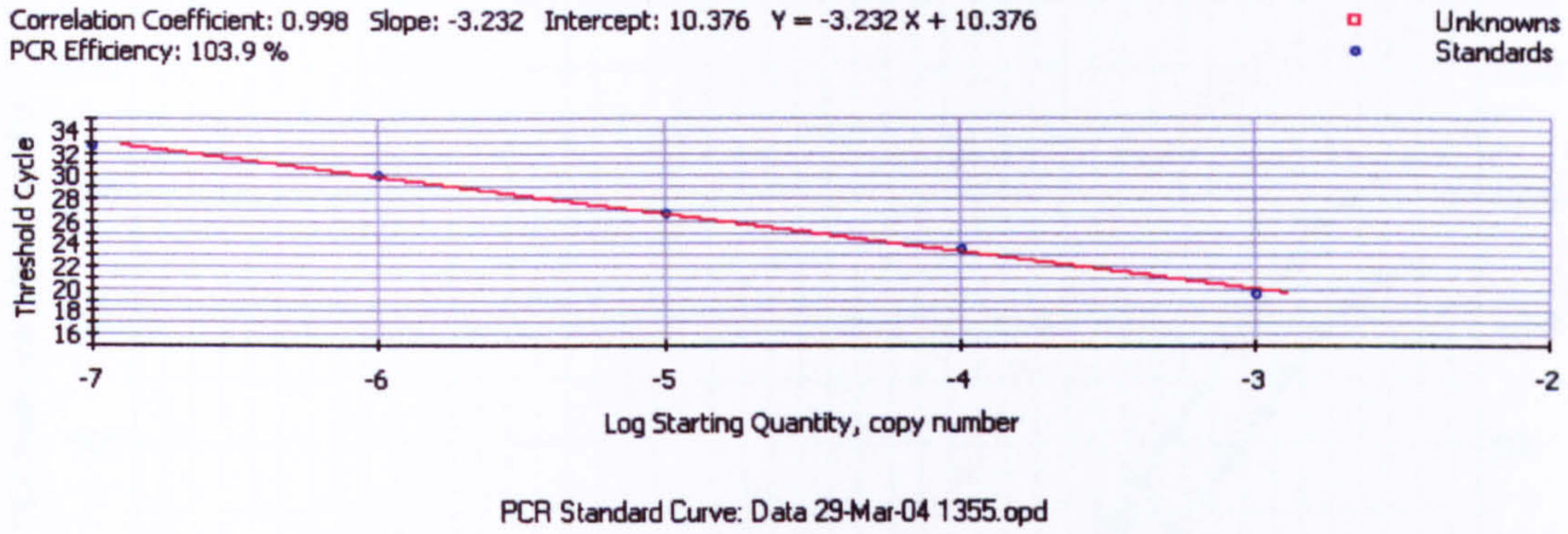


Figure 3.38 Standard curve of dilutions of ovine male DNA generated by plotting the Ct values of the 28S probe against the logarithm of the initial copy numbers




### 3.5.4.4 Dilutions of male ovine tissue in female ovine tissue

SRY DNA was detected in both samples containing 0.5% and 2.5% male DNA equivalent to 5 mg and 25mg of male brain tissue per gram of female brain tissue. Both negative and positive control samples produced expected threshold results.

Table 3.30 SRY probe threshold values for ovine male DNA contaminant

| Well | Ct | Template |
|---|---|---|
| C3 | 0.00 | 28s DNA (U1) |
| C4 | 35.1* | SRY +28s DNA (U2) |
| C5 | 32.8* | SRY +28s DNA (U3) |
| C6 | 26.7* | SRY DNA |
| C7 | 26.7* | SRY DNA |
| C8 | 0.00 | water |
| C9 | 0.00 | water |

Figure 3.39 SRY probe threshold values of DNA extracted from tissue samples

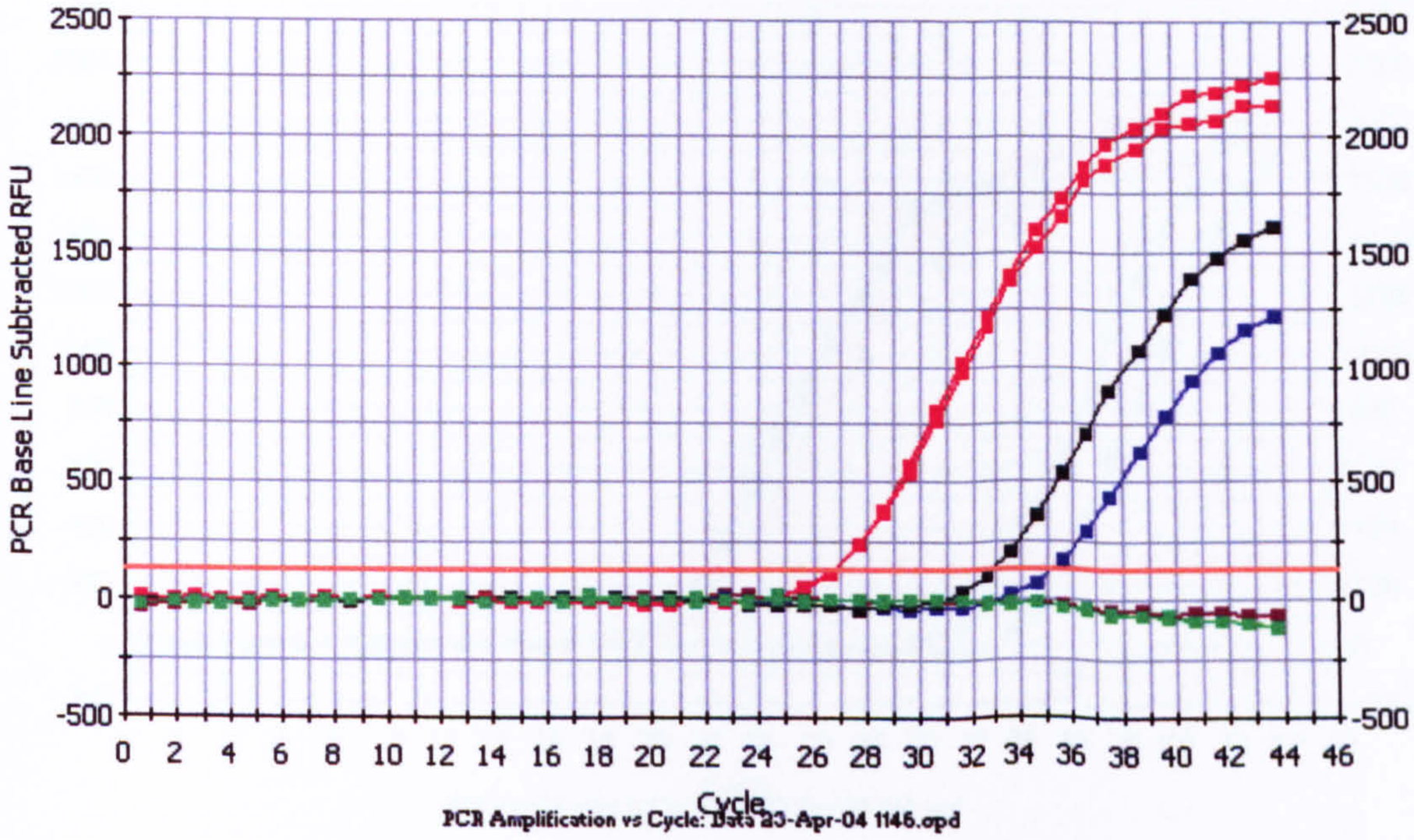
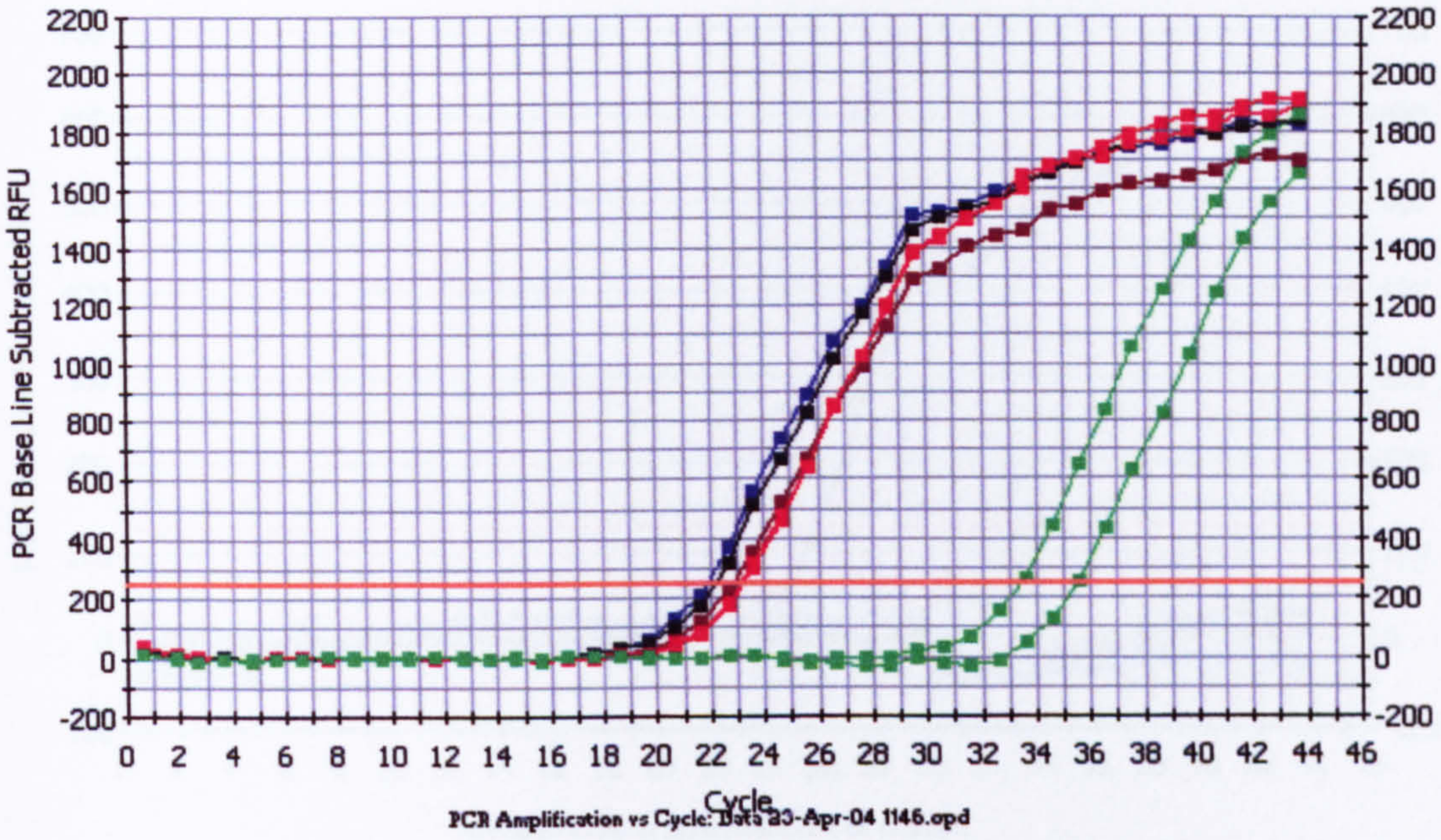



Table 3.31 28S probe threshold values for ovine male DNA contaminant and female DNA

| Well | Ct | Template |
|---|---|---|
| C3 | 22.9* | 28s DNA (U1) |
| C4 | 21.9* | SRY +28s DNA (U2) |
| C5 | 22.2* | SRY +28s DNA (U3) |
| C6 | 23.1* | SRY DNA |
| C7 | 23.2* | SRY DNA |
| C8 | 35.6* | water |
| C9 | 33.4* | water |

Figure 3.40 28S probe threshold values of DNA extracted from tissue samples




### 3.5.4.5 Detection of male ovine brain tissue in organs of a female ovine carcass

SRY-chromosome DNA was detected only in the positive control samples. The 28S rDNA was identified in all samples except for the negative controls.

Table 3.32 SRY probe threshold values for the selected organ samples

| Well | Ct | Template |
|---|---|---|
| D4 | 0.00 | Spleen |
| D5 | 0.00 | Spleen |
| D6 | 0.00 | Liver |
| D7 | 0.00 | Liver |
| D8 | 0.00 | Kidney |
| D9 | 0.00 | Kidney |
| E6 | 0.00 | water |
| E7 | 0.00 | water |
| E8 | 25.9* | Male ovine |
| E9 | 25.7* | Male ovine |

Figure 3.41 SRY probe threshold values of DNA extracted from organ samples

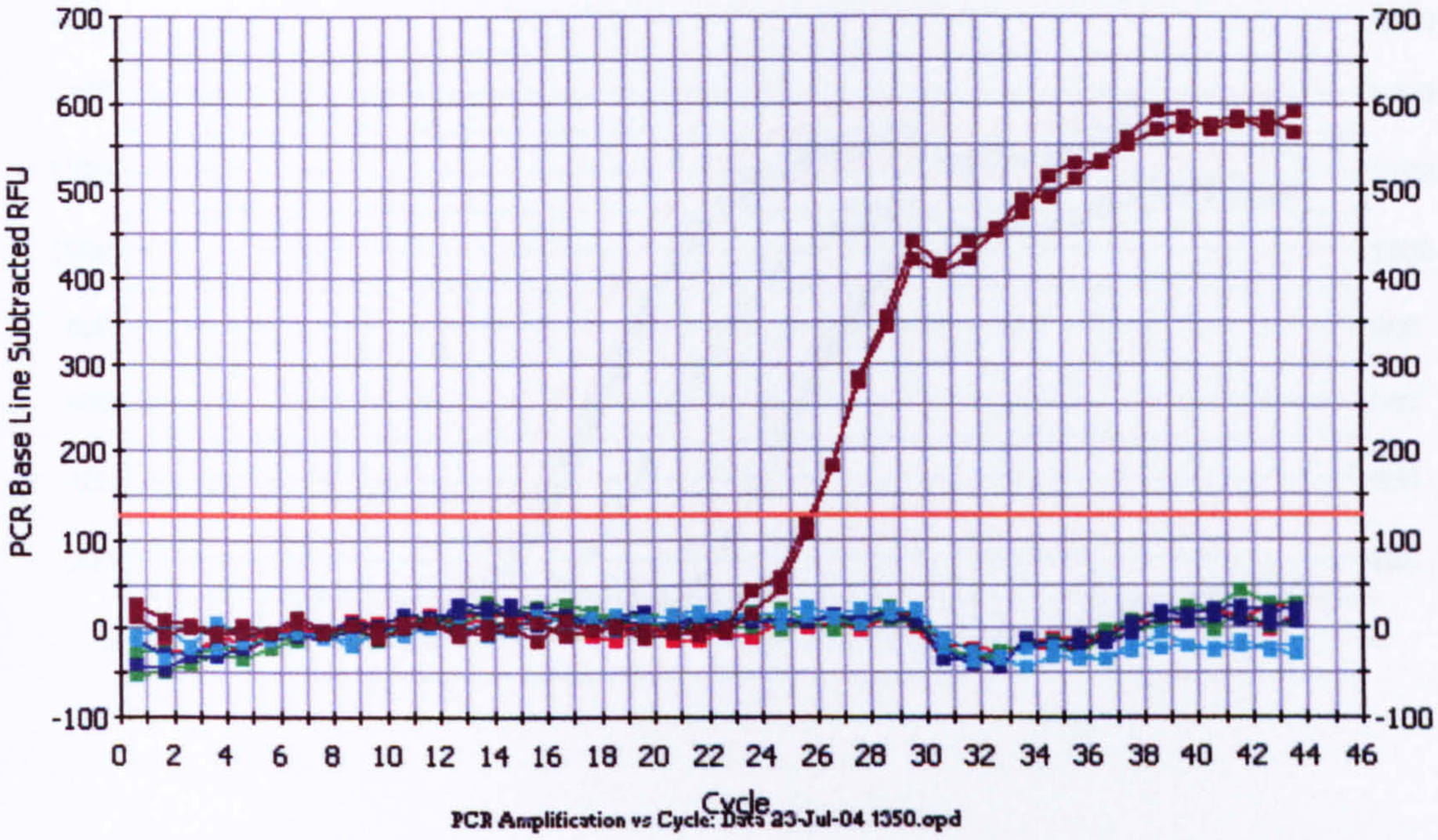
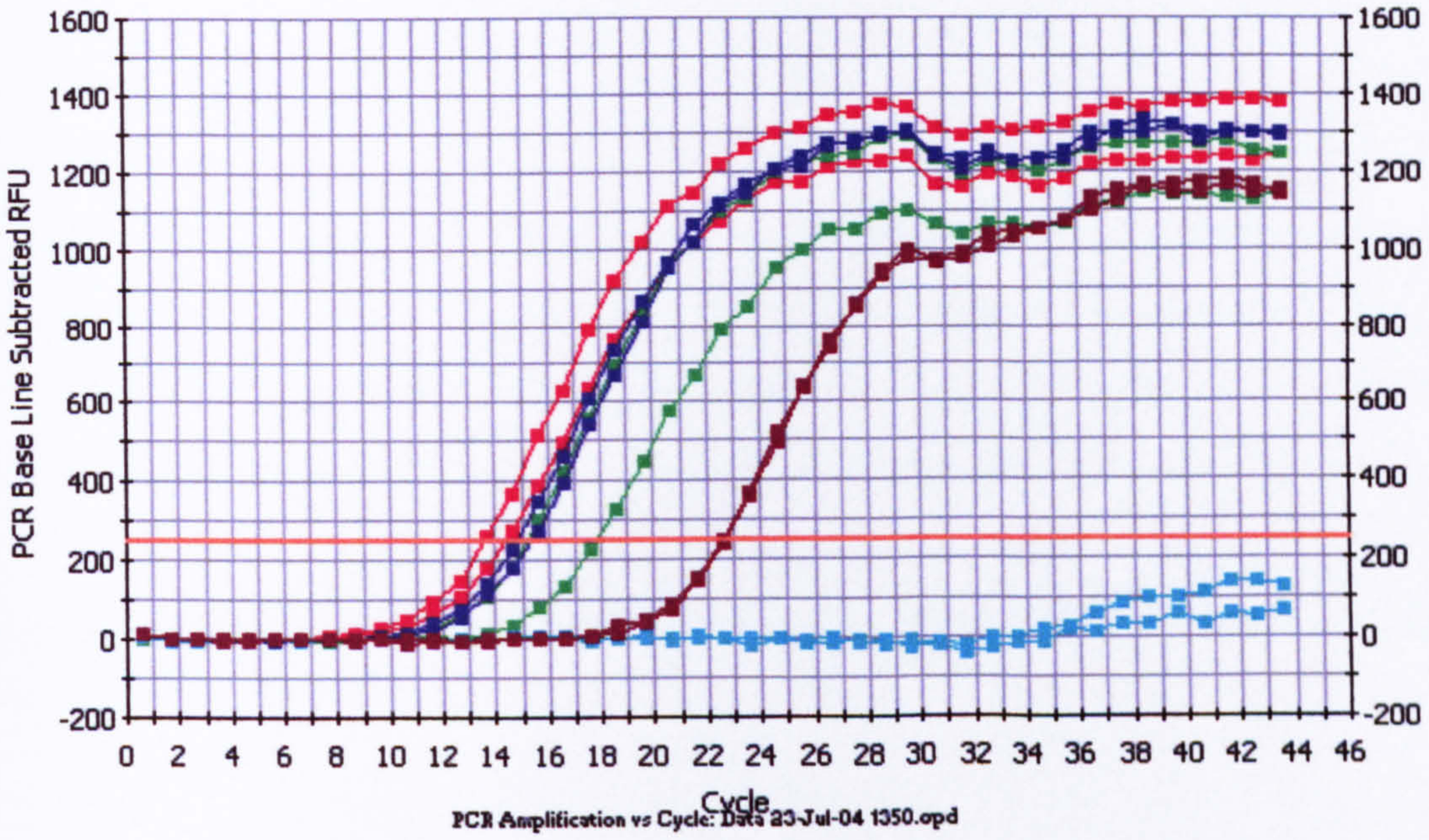



Table 3.33 28S probe threshold values for the selected organ samples

| Well | Ct | Template |
|---|---|---|
| D4 | 14.2* | Spleen |
| D5 | 13.3* | Spleen |
| D6 | 17.5* | Liver |
| D7 | 15.0* | Liver |
| D8 | 15.1* | Kidney |
| D9 | 14.6* | Kidney |
| E6 | 0.00 | water |
| E7 | 0.00 | water |
| E8 | 22.1* | Male ovine |
| E9 | 22.2* | Male ovine |

Figure 3.42 28S probe threshold values of DNA extracted from organ samples




## 3.5.5 Discussion

The real-time PCR assay was demonstrated to amplify ovine DNA at similar rates to that of bovine DNA in the samples analysed from each species. Furthermore, ovine SRY DNA from a male animal was demonstrated to be detectable in ovine tissues of female origin that were themselves identified by the use of the 28S rDNA present in both male and female tissues. The dilution of ovine male DNA demonstrated a detection limit of 0.05% or equivalent to 100-µg male brain tissue/ml of buffer. A subsequent experiment using artificially contaminated organs detected SRY DNA in samples containing the lowest prepared concentration of 0.5% male brain tissue in female tissues. The failure to detect the SRY DNA in the organ samples examined after captive bolt gun stunning and injection of brain tissue may indicate an absence of contamination above the limit of detection of the assay.

In some cases, 28S DNA was detected at low levels in the negative control samples. The explanation for this anomaly arises from use of batches of *Taq* polymerase in which contamination with 28S DNA is present originating from the strain of yeast from which the enzyme was obtained. The low levels present have previously been observed at similar threshold values (Dr. Chris Helps, University of Bristol, UK-Personal

communication), and are easily differentiated from the higher levels present in sample DNA.

## 3.6 INVESTIGATION OF PROTHROMBIN TIME ESTIMATION OF PLASMA AS AN INDIRECT TEST FOR BRAIN TISSUE EMBOLI IN BLOOD

### 3.6.1 Introduction

The development of coagulation abnormalities following severe head trauma has been extensively described (Kuo, Chou & Chio, 2004). Brain tissue contains the highest concentration of thromboplastin of any tissue in the body, and it is the release of this protein that activates the extrinsic pathway of the clotting cascade. Activation of the cascade results in the exhaustion of clotting factors present in the blood and would be expected to cause prolongation of prothrombin time measured in plasma obtained from such samples.

In this study, clotting times have been determined for plasma samples obtained from cattle and sheep within one minute of stunning, using determination of prothrombin time (PT time) as an accepted measure of clotting time.

While all animals suffered severe head trauma, only a small proportion were found to have brain tissue emboli in the venous circulation following analysis of samples by two methods of brain tissue detection (See 3.1.3). Prothrombin time values were investigated to determine the diagnostic value of this tool for predicting the presence of brain tissue emboli in blood from which plasma was obtained.

Currently, brain tissue fragments in venous blood samples, are detected using an ELISA for GFAP or by a complementary technique of microscopy and immunocytochemistry. These techniques although sensitive and specific are labour intensive, and may over-look some positive samples since each test can only examine a small sample aliquot of the collected blood. The use of PT time analysis would offer a quick and cost effective method for separating possible positive samples. Such samples could then be further analysed by more definitive methods.

### 3.6.2 Aims

- To investigate the use of prothrombin time estimation as a tool for identifying samples from sheep and cattle in which embolism of brain tissue into the blood occurred following stunning by captive bolt gun.

### 3.6.3 Methodology

#### 3.6.3.1 Estimation of prothrombin time of cattle plasma samples

Plasma was selected from twenty four cattle that were negative for brain tissue emboli and from six cattle in which brain tissue fragments or proteins had previously been detected by the combined technique of microscopy and immunocytochemistry or by ELISA. Seven plasma samples including a pre-stun and six post-stunning samples were available for analysis from each animal selected for inclusion in the study.

Table 3.34 Number of cattle selected in each group for analysis of plasma obtained pre and post-stunning

| Type of CBG and previous animal designation based on analysis for brain tissue emboli | Penetrating captive bolt gun | Non-penetrating captive bolt gun |
|---|---|---|
| Positive | 4 | 2 |
| Negative | 13 | 11 |

#### 3.6.3.2 Estimation of prothrombin time of sheep plasma samples

Plasma from eighty-two negative and from nineteen positive animals for brain tissue fragments or elevated levels of GFAP was included in the experiment. Seven plasma samples including a pre-stun and six post-stunning samples were available for analysis from each animal selected for inclusion in the study.

Table 3.35 Number of sheep selected in each group for analysis of plasma obtained pre and post-stunning

| Type of CBG and previous animal designation based on analysis for brain tissue emboli | Cartridge-activated captive bolt gun | Pneumatically-activated captive bolt gun |
|---|---|---|
| Positive | 9 | 10 |
| Negative | 38 | 45 |

### 3.6.4 Results

#### 3.6.4.1 Assay performance

Intra assay and inter-assay coefficients of variation of $\leq 5\%$ and $\leq 10\%$ were calculated.

### 3.6.4.2 Prothrombin time of cattle plasma samples

Table 3.36 Prothrombin times of plasma from cattle after use of non-penetrating and penetrating captive bolt guns that were found to be negative for brain tissue emboli

| | Plasma from cattle stunned by non-penetrating captive bolt gun | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| sample | Prothrombin times (s) | | | | | | | |
| | Pre-Stun | 1 | 2 | 3 | 4 | 5 | 6 | Mean Post-stun |
| 120 | 26 | 23 | 23 | 29 | 24 | 24 | 23 | 24 |
| 154 | 21 | 22 | 22 | 21 | 23 | 21 | 21 | 22 |
| 178 | 25 | 20 | 16 | 19 | 18 | 18 | 16 | 18 |
| 180 | 28 | 23 | 25 | 24 | 24 | 24 | 25 | 24 |
| 182 | 27 | 27 | 29 | 27 | 28 | 28 | 31 | 28 |
| 184 | 24 | 23 | 24 | 25 | 22 | 28 | 28 | 25 |
| 187 | 34 | 25 | 27 | 26 | 26 | 32 | 28 | 27 |
| 189 | 42 | 31 | 32 | 32 | 34 | 34 | 29 | 32 |
| 191 | 28 | 39 | 70 | 70 | 70 | 70 | 14 | 56 |
| 193 | 27 | 25 | 24 | 26 | 28 | 25 | 23 | 25 |
| 194 | 25 | 23 | 23 | 24 | 23 | 24 | 23 | 23 |
| mean | 27.9 | | | | | | | 27.6 |
| Variance | 32 | | | | | | | 101.06 |
| Plasma from cattle stunned by penetrating captive bolt gun | | | | | | | | |
| sample | Prothrombin times (s) | | | | | | | |
| | Pre Stun | 1 | 2 | 3 | 4 | 5 | 6 | Mean Post stun |
| 115 | 25.1 | 26 | 29 | 29 | 28 | 30 | 31 | 29 |
| 157 | 25 | 23 | 24 | 23 | 23 | 24 | 24 | 24 |
| 161 | 27 | 24 | 27 | 27 | 27 | 25 | 25 | 26 |
| 173 | 28 | 27 | 27 | 27 | 28 | 27 | 27 | 27 |
| 179 | 17 | 14 | 14 | 13 | 13 | 13 | 12 | 13 |
| 181 | 14 | 13 | 12 | 14 | 12 | 12 | 24 | 15 |
| 183 | 20 | 23 | 23 | 23 | 17 | 24 | 22 | 22 |
| 185 | 38 | 30 | 34 | 35 | 29 | 32 | 28 | 31 |
| 186 | 34 | 33 | 33 | 35 | 33 | 31 | 35 | 33 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 188 | 30 | 26 | 28 | 34 | 28 | | 31 | 29 |
| 190 | 28 | 24 | 23 | 24 | 28 | 25 | 24 | 25 |
| 192 | 26 | 25 | 25 | 26 | 25 | 25 | 25 | 25 |
| 199 | 22 | 21 | 24 | 23 | 24 | 24 | 22 | 23 |
| mean | 25.7 | | | | | | | 24.7 |
| variance | 42.5 | | | | | | | 33 |

Table 3.37 Prothrombin times of plasma from cattle after use of non-penetrating and penetrating captive bolt guns that were found to be positive for brain tissue emboli

| Plasma from cattle stunned by non-penetrating captive bolt gun | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 88 | 23 | 36 | 35 | 28 | 30 | 26 | 26 | 30.1 |
| 137 | 23 | 26 | 24 | 29 | 23 | 24 | 27 | 25.5 |
| mean | 23 | | | | | | | 27.8 |
| Variance | 0 | | | | | | | 10.8 |
| Plasma from cattle stunned by penetrating captive bolt gun | | | | | | | | |
| 65 | 25 | 33 | 34 | | | 32 | 43 | 35.5 |
| 67 | 29 | 31 | 35 | 35 | 43 | 30 | 34 | 34.6 |
| 129 | 30 | 30 | | | 39 | 20 | 32 | 30.25 |
| 133 | 27 | 34 | 31 | 31 | 30 | 30 | 25 | 30.1 |
| mean | 27.7 | | | | | | | 32.6 |
| variance | 5 | | | | | | | 8 |

### *3.6.4.2.1 Comparison of prothrombin times in pre-stun and post-stun samples*

A significant difference was observed between the pre and post-stunning sample means from samples in which brain tissue emboli was previously identified ($p=0.025$).

Table 3.38 Pre and post-stun sample prothrombin time mean values from positive bovine samples

| | Pre-stunning samples | Post-stunning samples | F value | Significance |
|---|---|---|---|---|
| Mean prothrombin time | 25.38 | 33.03 | 7.62 | $p \leq 0.05$ |

For samples negative for brain tissue emboli no significant difference was observed between pre and post PT times ($p=0.87$)

Table 3.39 Pre and post-stun sample prothrombin time mean values from negative samples

| | Pre-stunning samples | Post-stunning samples | F value | Significance |
|---|---|---|---|---|
| Mean prothrombin time | 26.80 | 26.48 | 0.03 | ns |

ns-not significant

### *3.6.4.2.2 Influence of captive bolt gun upon prothrombin time*

A general linear model with gun type and time as factors for samples from cattle positive or negative for brain tissue emboli indicated there was no significant difference between the type of captive bolt gun used ($p=0.49$ and $0.18$ respectively).

Table 3.40 Prothrombin time of plasma from cattle positive for brain tissue emboli after captive bolt gun stunning

| | Non-penetrating CBG | Penetrating. CBG | F value | Significance. |
|---|---|---|---|---|
| Mean prothrombin time | 28.21 | 30.20 | 0.52 | ns |

ns=not significant

Table 3.41 Prothrombin time of plasmas from cattle negative for brain tissue emboli after captive bolt gun stunning

| | Non-penetrating CBG | Penetrating. CBG | F value | Significance. |
|---|---|---|---|---|
| Mean prothrombin time | 28.21 | 30.20 | 0.52 | ns |

ns=not significant

#### *3.6.4.2.3 The presence of brain tissue emboli and prothrombin time*

No significant difference was found between the post-stunning prothrombin sample times from cattle positive or negative for brain tissue emboli. (t-test, p=0.068)

### 3.6.4.3 Prothrombin times of sheep plasma samples

Table 3.42 Prothrombin times of plasma from sheep after use of captive bolt gun stunning that were found to be negative for brain tissue emboli

| Sample number | Prothrombin times of plasmasamples (upper limit of 70-s) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pre-stun | 1st | 2nd | 3rd | 4th | 5th | 6th | Average post-stunning |
| 1 | 59 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 8 | 30 | 35 | 58 | 70 | 60 | 70 | 70 | 60.5 |
| 15 | 27 | 70 | 70 | 36 | | 70 | 36 | 56.4 |
| 22 | 26 | 63 | 37 | 26 | 65 | 37 | 39 | 44.5 |
| 29 | 26 | 36 | 65 | 70 | 35 | 38 | 30 | 45.6 |
| 36 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 43 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 50 | 28 | 32 | 56 | 31 | 70 | 70 | 70 | 54.8 |
| 57 | 45 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 64 | 22 | 12 | 70 | 70 | 70 | 70 | 70 | 60.3 |
| 78 | 28 | 30 | 63 | 70 | 70 | 70 | 70 | 62.1 |
| 85 | 26 | | 70 | 70 | 70 | 70 | | 70 |
| 92 | 30 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 99 | 30 | 13 | 70 | 70 | 70 | 70 | 70 | 60.5 |
| 498 | 29 | 29 | 38 | 40 | 44 | 62 | 70 | 47.1 |
| 505 | 30 | 21 | 36 | 56 | 28 | 63 | 41 | 40.8 |
| 533 | 30 | 21 | 36 | 56 | 28 | 63 | 41 | 40.8 |
| 547 | 27 | 70 | 43 | 33 | 30 | 25 | 28 | 38.1 |
| 582 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 589 | 70 | 33 | 39 | 70 | 70 | 70 | 70 | 58.6 |
| 603 | 70 | 28 | 70 | 70 | 70 | 70 | 70 | 63 |
| 610 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 617 | 26 | 25 | 47 | 29 | 29 | 29 | 70 | 38.1 |
| 631 | 26 | 70 | 70 | 70 | 35 | 36 | 41 | 53.6 |
| 645 | 31 | 35 | 70 | 70 | 70 | 70 | 70 | 64.1 |
| 659 | 33 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 673 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 680 | 31 | 67 | 70 | 70 | 70 | 70 | 70 | 69.5 |
| 694 | 31 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 701 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 708 | 33 | 31 | 37 | 30 | 29 | 34 | 36 | 32.8 |
| 715 | 30 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 729 | 26 | 49 | 53 | 70 | 70 | 70 | 70 | 63.6 |
| 743 | 30 | 30 | 46 | | 70 | 70 | 70 | 57.2 |
| 750 | 29 | 53 | 70 | 70 | 70 | 70 | 70 | 67.1 |
| 757 | 25 | 33 | 43 | 57 | 62 | 70 | 70 | 55.8 |
| 764 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 778 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 785 | 29 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 813 | 43 | 41 | 70 | 70 | 70 | 70 | 70 | 65.1 |
| 827 | 32 | 70 | 70 | 70 | 70 | 70 | 34 | 64 |
| 834 | 33 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 848 | 32 | 63 | 70 | | 70 | 70 | 70 | 68.6 |
| 855 | 29 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 862 | 34 | 36 | 70 | 70 | 70 | 70 | 70 | 64.3 |
| 869 | 32 | 70 | 70 | 70 | 61 | 56 | 62 | 64.8 |
| 876 | 29 | | 70 | 70 | | 70 | 70 | 70 |
| 883 | 39 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 967 | 37 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 974 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1016 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1037 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1044 | 70 | 70 | 70 | 70 | 70 | 70 | 54 | 67.3 |
| 1065 | 24 | 31 | 29 | 31 | 28 | 33 | 33 | 30.8 |
| 1072 | 24 | 70 | 70 | 67 | 55 | 48 | 41 | 58.5 |
| 1093 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1107 | 20 | 70 | 70 | 70 | 70 | 70 | 48 | 66.3 |
| 1128 | 27 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1142 | 38 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1149 | 31 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1156 | 25 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1184 | 31 | 70 | | 70 | 70 | 70 | 70 | 70 |
| 1191 | 29 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1205 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1219 | 44 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1226 | 32 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1289 | 24 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1296 | 10 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1303 | 20 | 70 | 70 | 70 | 70 | 54 | 41 | 70 |
| 1310 | 15 | 70 | 24 | 26 | 30 | 28 | 37 | 62.5 |
| 1317 | 23 | 70 | 29 | 43 | 70 | 70 | 70 | 35.8 |
| 1324 | 19 | 41 | 70 | 43 | 29 | 70 | 26 | 58.6 |
| 1331 | 28 | 70 | 70 | 26 | 33 | 70 | 28 | 46.5 |
| 1338 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 49.5 |
| 1352 | 27 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1359 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1366 | 25 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1373 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1387 | 23 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1394 | 24 | 70 | 70 | | | | 70 | 70 |
| 1401 | 26 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 1408 | 24 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| variance | 174.6 | | | | | | | 110.3 |
| mean | 32.1 | | | | | | | 63.2 |

Table 3.43 Prothrombin time of plasmas from sheep positive for brain tissue emboli after captive bolt gun stunning

| Cartridge-activated captive bolt gun | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Animal number. | Pre-stun sample number | Pre-stun prothrombin time | Post-stun prothrombin times (upper limit of 70-s) | | | | | | |
| | | | 1 | 2 | 3 | 4 | 4 | 6 | Mean |
| 74 | 512 | 27 | 70 | 43 | 33 | 30 | 25 | 28 | 38.17 |
| 75 | 519 | 28 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 111 | 771 | 39 | 38 | 70 | 70 | 70 | 70 | 70 | 64.67 |
| 114 | 792 | 30 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 130 | 904 | 25 | 49 | 66 | 70 | 70 | 70 | 70 | 65.83 |
| 132 | 918 | 27 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 155 | 1079 | 31 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 161 | 1121 | 29 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 169 | 1177 | 31 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| | mean | 29.66 | | | | | | | 65.41 |
| | variance | 16.25 | | | | | | | 108.7 |
| Pneumatically-activated captive bolt gun | | | | | | | | | |
| Animal no. | Pre-stun sample no. | Pre-stun prothrombin time | Post-stun prothrombin times (upper limit of 70-s) | | | | | | |
| | | | 1 | 2 | 3 | 4 | 4 | 6 | Mean |
| 115 | 799 | 36 | 70 | 70 | 70 | 70 | 50 | 70 | 66.67 |
| 118 | 820 | 24 | 27 | 33 | 70 | 70 | 70 | 70 | 56.67 |
| 121 | 841 | 30 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 129 | 897 | 31 | 57 | 70 | 70 | 70 | 58 | 45 | 61.67 |
| 137 | 953 | 29 | 37 | 70 | 70 | 70 | 70 | 70 | 64.5 |
| 143 | 995 | 32 | 70 | 49 | 70 | 70 | 70 | 70 | 66.5 |
| 163 | 1135 | 27 | 70 | 70 | 66 | 70 | 70 | 70 | 69.33 |
| 174 | 1212 | 35 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 178 | 1240 | 31 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 203 | 1415 | 25 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| | mean | 30 | | | | | | | 66.5337 |
| | variance | 15.33 | | | | | | | 20.12 |

### *3.6.4.3.1 Comparison of prothrombin times in pre-stun and post-stun samples*

A significant difference was observed between the pre and post-stunning sample means from samples both in which brain tissue emboli was previously identified and from samples that were negative for brain tissue emboli ($p<0.001$).

Table 3.44 Pre and post-stun sample prothrombin time mean values from positive ovine samples

| | Pre-stunning samples | Post-stunning samples | F value | Significance |
|---|---|---|---|---|
| Mean prothrombin time | 29.83 | 65.97 | 319.04 | $p\leq0.001$ |

Table 3.45 Pre and post-stun sample prothrombin time mean values from negative ovine samples

| | Pre-stunning samples | Post-stunning samples | t value | Significance |
|---|---|---|---|---|
| Mean prothrombin time | 32.2 | 63.3 | 18.17 | $p\leq0.001$ |

### *3.6.4.3.2 Influence of captive bolt gun upon prothrombin time*

A general linear model with gun type and time as factors indicated there was no significant difference between type of captive bolt gun ($p=0.721$).

Table 3.46 Prothrombin time of plasmas from sheep positive for brain tissue emboli after captive bolt gun stunning

| | Cartridge-activated CBG | Pneumatically-activated CBG | F value | Significance |
|---|---|---|---|---|
| Mean prothrombin time | 47.54 | 48.27 | 0.13 | ns |

ns=not significant

#### *3.6.4.3.3 The presence of brain tissue emboli and prothrombin time*

No significant difference was observed between post-stunning prothrombin times from sheep samples positive or negative for the presence of brain tissue emboli (t=-1.17)

### 3.6.5 Discussion

In this study, our aim was to investigate the activation of the clotting cascade in the blood of cattle and sheep after the application of captive bolt gun stunning. By determination of clotting times in plasma samples taken before and after stunning, the possibility of identifying animals in which brain tissue embolisation into the circulation has occurred, was explored. The use of prothrombin time to estimate clotting time was found to be an economical and readily available screening test for coagulation that gave consistent and reliable results in the bench tests that were conducted.

The results of the study indicate that in the sheep plasma assayed, PT times were generally prolonged in all post-stunning samples irrespective of whether brain tissue had been identified in the sample (See 3.1.3). However, in the cattle plasma assayed, a significant difference was observed between pre-stun and post-stun samples only from cattle previously designated as positive for brain tissue emboli. No significant difference was observed between the calculated means of pre and post-stun samples from cattle in which brain tissue material was not detected. These observations indicate that measurement of PT time in cattle plasma might be useful for identification of cattle in which brain tissue embolisation had occurred following stunning. In contrast, the

results from assay of sheep plasma suggest that determination of PT time would not be helpful in similar investigations.

The uniformly prolonged PT times observed following assay of sheep plasma may be the result of tissue factor release from tissues traumatised by the passage of the bolt after stunning. While similar abnormalities in clotting time have been documented in human studies of clotting following head trauma (van der Sande, et al., 1978), the activation of the clotting cascade within ten seconds of head trauma demonstrated in these experiments has not been recorded. The prolonged period between injury and blood sampling in studies that rely on collection of plasma from victims of accidental head trauma, may account for the lack of data on this observation. It is possible that these findings of rapid activation of the clotting cascade may have application to research areas separate from stunning and slaughter of livestock. The activation of the clotting cascade is known to be an important factor in the prognosis of human victims of head trauma (Simpson, Speed & Blumbergs, 1991).

Previous estimates of coagulation abnormalities following head trauma of humans have ranged from 3% to 72 % (Kuo, Chou & Chio, 2004; Selladurai et al., 1997). In this study elevated coagulation scores beyond sixty seconds in length were observed in 80% of post-stunning samples taken from sheep. Only 3% of cattle samples taken post-stunning demonstrated PT times beyond sixty seconds. The wide range of PT times observed in these and other investigations, suggests the influence of several factors including, the method of sampling, processing and assay of plasma, the species studied and the type and severity of the head injury.

While it is likely that brain tissue emboli in the blood may release large amounts of the clotting activator thromboplastin, the activation of the cascade in sheep appears to happen regardless of the presence or absence of brain tissue fragments in the blood. Any additional activation of the extrinsic clotting pathway by release of clotting activators from brain tissue fragments may be concealed by the larger effect of tissue factor release from injured tissues. A consistent effect of head trauma upon prolonged clotting time is less apparent in the cattle samples where a difference between pre and post PT times is detected only from the samples previously designated as positive for brain tissue embolism. This finding may be due to the additive effect on PT time of thromboplastin released from brain tissue fragments into the blood. Comparison of the

mean post-stunning PT times demonstrates a significant difference between bovine samples negative or positive for brain tissue embolism. This suggests that in cattle, measurement of PT time in plasma may be useful for identifying samples containing brain tissue fragments.

The observed difference in PT time between sheep and cattle plasma samples may relate to the greater volume of blood in cattle into which thromboplastin is released from similar levels of tissue damage. This dilution effect may prevent PT times from being prolonged in cattle samples unless brain tissue fragments are also present to prolong PT times even further. The prolonged prothrombin times observed in this study were detected in a small sample population of animals that were identified as positive for brain tissue embolism. A larger population of positive animal samples would increase the confidence in these results. Furthermore, the analysis of plasma by other measures of clotting activity including D-dimer levels might reveal additional correlations between brain tissue emboli and coagulopathy.

# CHAPTER 4 DISCUSSION AND CONCLUSIONS

## 4.0 INTRODUCTION

Since brain tissue fragments were first reported in the pulmonary arteries of a cow at post-mortem following the use of an air-injection pneumatically-activated CBG (Garland, Bauer & Bailey, 1996), a number of research projects have been undertaken to substantiate these findings. In addition, investigations have examined the risks associated with stunning and slaughter and carcass contamination with brain tissues (Anil et al., 1999, 2001; 2002; Schmidt et al., 1999). These studies made significant contributions to the body of knowledge on this subject and led to recommendations and measures being taken on stunning and slaughter of livestock by a European Scientific Steering Committee (SSC) (EC, 2002). In a report, the SSC concluded that the practice of pithing after penetrating CBG stunning and the use of penetrating air-injection CBGs would carry unacceptably high risks of causing CNS tissue embolism (EC, 2002). The use of these stunning methods was later banned by revision of EU legislation (EC Regulation 999/2001). Based on the research data available at the time the Spongiform Encephalopathy Advisory Committee (SEAC) in the UK (SEAC, 1998) recommended that further research on stunning and carcass contamination with CNS tissues should be undertaken.

In this series of experiments a number of important questions have been explored and in some cases answered. In addition, the work has indicated gaps that remain in research on this subject and potential directions and methods that might be employed in future experimental work. The main questions that this work has attempted to address include:

- Which stunning methods currently in use/or available in the UK carry a risk of brain tissue embolism?
- What is the frequency of brain tissue entry into the venous circulation after stunning by captive bolt gun in cattle and sheep?
- Can brain tissue fragments and proteins disrupted by captive bolt gun stunning of cattle and sheep penetrate the pulmonary capillary filter and enter the arterial circulation within the time-frame of commercial stunning and slaughter?
- How much brain material enters the venous blood of sheep after captive bolt gun stunning?

- What are the potential venous drainage routes of emboli from the head following the use of captive bolt gun stunning?
- What organs of the carcass may be contaminated with brain tissue emboli after stunning?

# 4.1. DETECTION OF BRAIN TISSUE CONTAMINATION IN BLOOD AND OTHER TISSUES USING AN ELISA AND A COMBINATION OF MICROSCOPY AND IMMUNOCYTOCHEMISTRY

## 4.1.1 JUGULAR VEIN BLOOD SAMPLING FOLLOWING STUNNING

This study has confirmed the potential of captive bolt gun stunning devices currently in use in the UK to cause entry of brain tissue fragments into the venous circulation after stunning. The public health risk associated with small levels of brain tissue or brain protein contamination of the venous blood is still unknown. In the absence of reliable infectious dose data applicable to humans, it must be assumed that even minute quantities of BSE infected tissue in meat pose an unacceptable risk to the consumer. The results of experiments to determine the frequency of brain tissue embolism following stunning in both cattle and sheep allows more accurate estimation of the risk of carcass contamination and can be used to influence policies on the continued use of these mechanical stunning methods in countries in which a theoretical risk of BSE transmission to humans exists.

Previous studies examining brain tissue embolisation after head trauma have suggested rupture of the dorsal sagittal sinus as the most likely point of entry of brain tissue (Miyaishi et al., 1994), while other reports indicate that the rupture of blood vessels in the cranium may not be necessary for brain tissue proteins to enter the circulation (Mussack et al., 2003). It seems likely that different factors are involved in the entry of brain tissue fragments to the circulation, in contrast to brain tissue proteins that may enter the venous circulation following relatively minor disruption to the blood brain barrier. It is possible that any stunning method will run the risk of causing entry of small brain proteins including the prion protein into the venous circulation, as minor head trauma ,including that which might happen as the stunned animal strikes its head on the stunning box floor, appears unavoidable. However, by limiting the potential for more significant levels of contamination by way of brain tissue fragments in the blood, the risk of dangerous levels of TSE infectivity reaching the consumer may be reduced.

The presence of two mechanisms of entry of brain proteins and of brain tissue fragments into the venous return offers an explanation for the disparity in the findings of the two analytic methods employed in these studies. The ELISA results of some positive cases may detect GFAP that has leaked across a damaged blood brain barrier

but in which insufficient trauma has occurred to blood vessels to allow entry of fragments of brain tissue detectable by microscopy and immunocytochemistry. Although the latter technique uses a combination of analytic methods to identify positive samples, any brain tissue fragments in a sample must be large enough to first be tentatively identified by microscopy before being positively identified by antibody staining.

A previous study of GFAP blood concentrations of brain proteins conducted using samples taken from human subjects soon after head trauma found lower levels of GFAP than S-100β proteins. The authors suggested that enzymatic degradation of GFAP in blood might offer an explanation for this observation (Missler et al., 1999). If significant degradation of GFAP does occur in blood, it may offer an additional explanation for the absence of elevated levels of GFAP in some samples that were found to contain brain tissue fragments.

Examination of the results of the frequency of brain tissue embolism study in cattle demonstrates a significant difference in the numbers of individual samples identified as positive by either CBG stunning method, although the overall numbers of positive animals found in each group is not significantly different. These results suggest that the penetrating CBG is likely to produce more positive samples than observed in samples obtained from cattle after use of the non-penetrating CBG. This finding is consistent with earlier predictions outlined in a report by the European Commission (EC, 2002) that the penetrating CBG would be more likely to cause brain tissue emboli to enter the circulation than the less invasive non-penetrating gun. However, the discovery that the non-penetrating captive bolt gun can also induce brain tissue embolism is an important deviation from the risks outlined in the report by the EC in which the risk of brain tissue embolism after non-penetrating CBG stunning is described as negligible (EC, 2002).

The results of the parallel study in sheep produced significantly higher frequencies than those observed in the cattle study and seem to suggest a strong case for reviewing the use of these stunning methods in this species especially in light of the availability of an effective alternative method by way of electrical stunning. Comparison of the number of positive sheep identified in each CBG group did not indicate a significant difference between each group while examination of the individual samples identified as positive

in each group also did not suggest a difference between the numbers of positive samples and the type of CBG used.

A comparison of the frequency of brain tissue embolism after stunning in cattle and in sheep suggests possible mechanisms of entry of brain tissue fragments into the venous circulation. The frequency of entry of brain tissue fragments into the venous circulation would appear to be between 5 and 10 times greater in sheep than in cattle despite reported findings of similar brain tissue and vessel damage in both species after captive bolt gun stunning (Farag, 2002; Biggins, 2003). These findings may indicate that brain tissue damage and haemorrhage are secondary factors to a more important determining variable that may be intra-cranial pressure. An investigation of the effect of reducing intra-cranial pressure at CBG stunning might provide solutions to effective stunning while reducing the risk of brain tissue emboli.

The investigation of brain tissue emboli and electrical stunning although not conclusively excluding the possibility of entry of brain tissue fragments into the circulation suggests this would be a rare event if it occurred at all. Further investigations on a larger sample group of cattle in which both pre and post-stunning samples were taken and analysed might resolve the anomaly of the single positive sample detected in this study. It is probable that the elevated levels of GFAP detected in the single positive sample represented the outcome of gliosis with increased production and release of GFAP into the circulation, a finding that is previously recorded in the literature (Uyeda, Eng & Bignami, 1972; Eng, Ghirnikar & Lee, 2000). Although the absence of a pre-stun sample in this experiment prevents confirmation of this explanation for the result, some precedent is set by a previous sample tested as part of the brain tissue embolism study in sheep in which all seven samples including that of the pre-stun sample demonstrated elevated levels of GFAP. These samples were accordingly designated as negative for the presence of brain tissue emboli despite the relatively high concentrations of GFAP in the samples detected by ELISA.

The results of the parallel studies in cattle and sheep indicate the need for revision of the ranking of stunning methods and potential for causing brain tissue emboli previously proposed by the EU Scientific Steering Committee (EC, 2002). Based on the combined results from both investigations a revised ranking of stunning methods in descending order of reducing risk is as follows.

- Air-injection pneumatically activated CBG
- Cartridge-activated penetrating CBG
- Pneumatically-activated penetrating CBG
- Cartridge-activated non-penetrating CBG
- Electrical stunning (absent or negligible risk)

This ranking based solely on the frequency results obtained in each study ignores several relevant factors including, the species in which the stunning methods were used and evaluated, throughput and local method of application and expertise of slaughtermen. With the exception of electrical stunning all of the listed stunning methods have been demonstrated to potentially cause brain tissue emboli in some animals following the application of stunning. An accurate estimation of the relative safety of each method as regards brain tissue contamination of the carcass would require further study of each stunning method in each relevant species.

The public health concerns of the use of each of these stunning methods were considered in a recent report by the European Food Safety Authority (EFSA, 2004). In this report, penetrating captive bolt stunning is described as carrying a risk of haematogenous contamination of tissues as well as the potential to cause air-borne contamination of the operator, environment and hide of the stunned animal. Non-penetrating CBG stunning was reported to carry a risk of embolism of brain tissue material while electrical stunning was described as having no public health concerns for the spread of BSE.

Completely preventing entry of brain proteins and tissue fragments into the venous circulation after stunning is unlikely to be possible since minor head trauma such as might occur if the animal were to strike it's head on the floor after stunning might itself carry a small risk of embolism. Nevertheless, the results of this study indicate that a wide range of frequencies of brain tissue embolism are possible dependant upon variables that include the type of captive bolt gun used for stunning and the size and species of animal to be stunned. This suggests that it may be possible by modified or novel stunning methods to minimise the likelihood of embolism to very low levels which if combined with testing of animals for BSE might reduce the risk of BSE transmission to the consumer by this route to negligible levels.

### 4.1.2 AORTIC BLOOD SAMPLING

The potential of fragments of brain tissue to disseminate in the carcass has been the subject of considerable debate. Previously brain tissue emboli have been detected in the right side of the heart or lodged in the pulmonary arteries, leading some to speculate that passage beyond the lungs was unlikely (Bradley, 2002). A study using marker bacteria has demonstrated wide dissemination of the bacteria in the carcass after captive bolt stunning (Prendergast et al., 2004). Although useful this study provided little information on the dynamics and potential for brain tissue fragments to disseminate in the carcass since the biological composition and size of brain tissue fragments and bacteria must be very different.

In previous studies conducted by the author a prepared brain tissue suspension was stained with a blue dye and then introduced into the pulmonary arteries of abattoir specimens of bovine lungs. These simple experiments demonstrated that in these isolated organ specimens, passage through the lungs and into the pulmonary veins was possible (Coore, unpublished trials). These crude demonstrations however were not transferable to the live animal in which muscle and vessel tensions and permeability are likely to be very different. Nevertheless, these trials were useful in formulating the techniques that were finally used in the aortic sampling experiments conducted in sheep and cattle.

The sampling of the aortic blood following exogenous introduction of brain tissue fragments to the venous circulation has demonstrated in sheep that the passage of small brain tissue fragments through the pulmonary capillaries is possible and furthermore has suggested the size of particles small enough to pass through and enter the aortic circulation.

In aortic blood samples obtained from cattle, elevated levels of GFAP were detected in samples from three animals by ELISA while all samples were found negative by microscopy and immunocytochemistry. It is possible that brain tissue fragments were also present but were not identified in the prepared Cytoblocks since less than one percent of each blood sample was actually examined for the presence of brain tissue fragments (Prof. S. Love, Department of Neurosciences, Frenchay Hospital, Bristol, UK-personal communication). An explanation for finding brain tissue fragments and

proteins in sheep samples as opposed to brain proteins alone, in cattle samples may be due to the proportionately greater volume of blood in the bovine and the consequent much greater opportunity for missing positive samples as a result of greater dilution effects in cattle than in sheep. In any event, it seems clear that the prion protein ($PrP^{sc}$) in a BSE infected animal would pass into the aortic blood after stunning whether associated with larger fragments of brain tissue or perhaps simply as a protein in suspension. Once present in the aortic blood, brain tissue fragments or proteins may potentially be carried to all areas of the carcass including to muscles.

The timing of passage through the lungs was another important aspect of this study, since in order to present a risk to the consumer of beef, brain tissue would need to access the aortic circulation before the circulation was halted by cardiac arrest due to exsanguination. In samples positive for elevated levels of GFAP or in those samples containing visible fragments of brain tissue, positive blood samples were found within a minute of stunning and injection of brain tissue suspension. This time frame is well within that which would allow contamination of the carcass to occur in the real abattoir situation. During commercial stunning and slaughter of livestock there is an interval of between thirty and ninety seconds between stunning and exsanguination achieved by cutting of major blood vessels in the neck or thorax. During this interval the stunned animal may be shackled and hoisted prior to exsanguination (sticking) depending upon the slaughter practice followed (in some abattoirs the animal may be exsanguinated without hoisting). This stunning to sticking interval during which the heart is still beating and the circulation is intact allows a window of opportunity for dissemination of brain tissue emboli through the lungs and into other tissues of the carcass. Clearly in abattoirs with prolonged stunning to sticking intervals one would expect an increased risk of passage of brain tissue emboli through the lungs and subsequent contamination of the carcass with brain material.

Lymphocytes have been implicated in the peripheral pathogenesis of prion diseases following experimental transmission of BSE in sheep using infected blood and buffy coat (Hunter et al., 2002) and also following a case of vCJD in an individual as a result of a blood transfusion from a sub-clinically infected donor (Ironside & Head, 2004). Plasma is also suspected to contain lower but still detectable levels of infectivity (Ironside & Head, 2004). If blood from a BSE infected animal may already carry sufficient infectivity to cause infection in some susceptible individuals, then brain tissue

emboli may create by an additive effect a greater level of risk to more people in whom the species barrier might otherwise have prevented infection. The study has been successful in going further towards demonstrating the potential for carcass contamination by CBG stunning but still leaves unanswered questions as to which organs are most likely to be contaminated and the quantity of brain tissue that might disseminate in the carcass.

The vertebral venous plexus is a complex system of veins that assist in the drainage of blood from the head. By occluding the jugular veins of anaesthetised sheep, an investigation of alternative drainage routes from the head was made, using GFAP as a marker for brain tissue. It was hoped that occlusion of the jugular veins might force brain tissue fragments introduced into the cerebral sinuses to exit the head by way of the vertebral veins. Alternatively, such brain tissue fragments might be forced to drain from the head through the lymphatic system by the change in pressure gradient in the venous system produced by blocking jugular drainage. The negative results obtained from the aortic blood samples and from organ samples including the cervical lymph nodes were inconclusive. Although laboratory trials with lymph node specimens and injected brain tissue suspension suggested that the GFAP ELISA might be sensitive enough to detect the presence of brain tissue in organs (Refer to 3.1.3.9.2.2), the high concentration of injected brain tissue used in these bench tests probably accounted for the success in detection. In the experiments conducted on the live animal, if brain tissue contamination did occur, the levels of contamination would probably have been many orders of magnitude lower making detection by this method impossible. The sheep injected with brain tissue suspension in these experiments were lying in lateral recumbency on the table that should have caused some blood to drain by way of the vertebral plexus (Coore et al., 2004[b]). However, it is possible that the time required for circulation of brain emboli down through the plexus and into the posterior vena cavae before return to the heart and lungs may be far in excess of that available between the act of stunning, sticking and exsanguination. It seems unlikely that the lymphatic system contributes a significant risk of brain tissue dissemination within the carcass. Nevertheless, particles injected into the cerebral sinuses of anaesthetised animals have been demonstrated to be transported from the cerebral sinuses by this route (Kida, Pantazis & Weller, 1993), although the time frame for this transport described in these experiments exceeds that which occurs within the abattoir situation at stunning and slaughter.

### 4.1.3 ORGAN SAMPLING

The sampling of organs and analysis by the methods of ELISA and the combined methods of microscopy and immunocytochemistry were inconclusive since the negative results obtained might indicate an absence of organ contamination or simply indicate levels of contamination below the detection limit of each method of analysis. These trials suggested the need for the application of more sensitive detection methods and also for methods that avoid the need to sample small areas of organs while leaving the greater volume of tissue un-tested.

### 4.1.4 VALIDATION

The sandwich ELISA for GFAP used in these experiments was previously validated in experiments examining spinal cord tissue contamination of carcasess (Helps et al., 2002). Although the assay protocol was essentially unchanged in these experiments, the application of the assay to blood samples rather than to swabs required that the sensitivity and specificity of the assay was re-evaluated. The validation studies demonstrated that this assay based on commercially available antibodies is robust and produces consistent and reliable results. The sensitivity of the assay for GFAP was 0.5ng/ml that compares favourably with previous estimates of the limit of detection of similar assays (Helps et al., 2002; Schmidt et al., 1999). However, it is acknowledged that the limit of detection of this assay is many orders of magnitude higher than other ELISAs for GFAP that quote sensitivities as low as 5pg/ml (Petzold et al., 2004). Although assays offering greater detection sensitivities might appear to offer improved methods of detecting brain tissue contamination of the carcass, such assays may only result in greater detection of background GFAP levels and lead to further confusion as to the validity of the results.

The validation studies of the complementary technique of microscopy and immunocytochemistry confirmed the findings of previous trials that used this method and in fact demonstrated a greater degree of consistent sensitivity than previously reported (Love et al., 2000).

## 4.2 ANATOMICAL INVESTIGATION OF VENOUS CEREBRAL DRAINAGE

### 4.2.1 VASCULAR CASTING

The vascular structures demonstrated in each of the prepared casts indicate an essentially similar structure in both bovine and ovine specimens. The sinuses of the head communicate directly or indirectly with both the jugular veins and with the vertebral venous system.

The internal vertebral plexus is the most prominent vascular structure of the vertebral venous system demonstrated by the resin casts. This system has clearly developed as an important drainage portal for blood from the head. This observation confirms that of previous workers who identified the vertebral venous plexus as a collateral venous pathway and a potential conveyer of infection and emboli to the vertebrae from the head (Batson, 1940; Smuts, 1977).

An examination of the bovine specimens suggests that the vessels of the calf specimen are relatively larger than those of the adult, although the vascular systems appear to be of similar structure and complexity. The ovine specimen demonstrates a system of vessels of generally smaller bore than in the bovine specimens consistent with the relative size of the animals.

The vertebral plexus represents an alternate route, paralleling the caval system in transporting blood back to the heart via various anastomoses with the systemic as well as the visceral veins. It provides a bypass for the caval, portal and pulmonary systems and in view of the continuity with the dural sinuses inside the cranial cavity and the absence of valves in both systems, it allows blood to flow in either direction dependant upon the pressure gradient within the system (Goschal, Koch & Popesko, 1981). This by-pass facility may be an important factor in the potential dissemination of brain tissue emboli by this complex system of veins. It suggests that brain tissue emboli from the head might be transported to visceral organs without passage through the lungs, that may act as an important filter system for emboli transported via the alternative jugular route.

### 4.2.2 DISSECTION OF THE OVINE HEAD AND NECK

This study demonstrated that the use of dissection alone without the combined use of resin injection of the venous system is difficult, due to the surrounding bony and soft tissues that protect the cerebral sinuses and vertebral plexus. However, the method does offer the advantage of demonstrating the relationship of the drainage system to the surrounding soft tissues such the vertebral vein to the transverse canal of the cervical vertebrae. In contrast, the technique of resin casting and maceration of tissues does not allow easy observation of this relationship, since all tissues are dissolved in the cast preparation.

In this dissected specimen the vertebral vein was visualised and measured accurately without the use of prior resin injection that may have otherwise exaggerated the diameter of the vessel. In future similar anatomical studies, the combined used of resin injection and dissection may allow a more detailed investigation of the vertebral venous system.

## 4.3 RADIOGRAPHIC STUDY OF VENOUS CEREBRAL DRAINAGE

The potential role of the vertebral venous system as an additional drainage route for brain tissue emboli in suspension in the venous blood was demonstrated radiographically in this series of experiments.

In addition, the postural effect on cerebral venous drainage demonstrated in previous studies conducted in other species (Dilenge & Perey, 1973; Zippel, 2001) was confirmed in this study of sheep. In the horizontal recumbent position, blood flow appears to drain from the head by the vertebral venous plexus as well as by both jugular veins. In contrast, in the vertical 'head down' position, blood flow returns to the body exclusively by the jugular route. Previous workers have suggested variations in pressure in the cerebrospinal fluid as the explanation behind this observation (Dilenge and Perey, 1973). This factor may have particular significance to captive bolt gun stunning since the act of stunning is likely to cause rapid and significant elevations in pressure that might result in the preferential use of unexpected drainage pathways including that of the vertebral plexus.

The practical implications of the effects of posture upon transport of brain tissue emboli may mean that in abattoirs with prolonged stunning to hoisting intervals there may be a greater risk of dissemination of emboli by way of the vertebral plexus. Blood draining through the vertebral plexus will avoid the filtration effects of the pulmonary capillaries and might carry brain tissue fragments to peripheral tissues of the carcass. The longer that an animal remains on the stun box floor and the holding cradle before hoisting, will allow a greater volume of blood to drain from the head through the vertebral venous system. This will effectively increase the risk of brain tissue emboli dissemination by this route. This factor may create a further imperative for reducing stunning to hoisting intervals since slow shackling and hoisting will also increase stunning to sticking intervals with concomitant adverse effects upon welfare. Abattoirs with rapid stunning to sticking intervals that also hoist animals before exsanguination will substantially reduce risks of brain tissue dissemination in the carcass by the vertebral venous system.

## 4.4 SEPARATION AND QUANTIFICATION OF EMBOLI BY FILTRATION

This small pilot study has demonstrated a new and practical method of recovering brain tissue fragments in blood samples, with the added advantage of providing additional data on the weight of brain tissue that may enter the circulation after stunning. Although leukocyte filters have been proposed and used for this purpose in an earlier study (Anil, Krailadsiri & Seghatsian, 2002), the use of the pre-filter to capture brain tissue fragments, as opposed to the leukocyte filtration component is an innovation that has several advantages over this use of the filtration apparatus. The main such advantage lies with the ease of recovery of the captured tissue, since the leukocyte filters themselves are difficult to open, and back-flushing with saline to recover the tissue is likely to recover only fractions of the brain tissue material. The use of the pre-filter however, is cost and time effective allowing the processing and analysis of large volumes of collected blood. It avoids the necessity for sampling small volumes of the blood collected, as was the case with the method of separation by centrifugation (Refer to 2.1.8.1). Secondly, the method avoids confusion over the nature of the captured material since the tissue captured in the filters can be visualised and positively identified by microscopic and immunocytochemical analysis. Brain tissue proteins including GFAP and Syntaxin 1B will pass through the filter into the filtrate and can be detected by application of a suitable ELISA.

The study has provided the first experimental evidence of the weight of brain issue that enters the circulation after captive bolt gun stunning. This information is a critical determinant of any risk analysis of captive bolt gun stunning, and the potential for carcass contamination with infective prions. The finding of an average weight of 1-g of brain tissue in the jugular venous blood compares favourably with the weights of loose brain material collected from the bolt hole of bovine and ovine skulls after captive bolt gun stunning (Farag, 2002, Biggins, 2003). The infective oral dose of BSE infected brain tissue to humans is unknown, however, studies in animals estimate a minimum oral dose of 50-μg to 500-mg of infected brain tissue (Love et al., 2000). Using these estimates of brain tissue infectivity, one-gram of BSE infected brain tissue would contain between 2 and 20,000 times the amount necessary for transmission of infection by the oral route.

Previous methods of analysis have been unable to provide an accurate estimate of the amount of brain tissue that may enter the venous circulation due to reliance on sampling of small volumes of collected blood samples. Estimates of up to 10-g of brain tissue have been made based upon the amount of brain material that can be dislodged by the penetrating captive bolt gun (Anil et al., 2002). However direct measurement of the amount of brain material present in the circulation after stunning has not previously been described. The European Food Safety Authority (EFSA) recently published a report on BSE risk from dissemination of brain particles in blood and carcasses (EFSA, 2004), in which new research findings in the field are discussed and described, including many of the findings from this study. In this report by EFSA, the need for further research on quantification of the contamination of organs with brain tissues is reiterated.

In future studies, refinements to the use of the filtration method might include the use of filters with smaller pore sizes as well as improvements in the analysis of the captured tissue. Such new analytic methods might allow an accurate estimation of the proportionate weight of brain tissue, as opposed fibrin and debris captured in the filter.

## 4.5 POLYMERASE CHAIN REACTION FOR DETECTION OF BRAIN TISSUE CONTAMINATION OF THE CARCASS

The aim of this study was to determine the extent of organ contamination with brain tissue that may occur following the use of captive bolt gun stunning of sheep. By injecting quantities of brain tissue into the jugular blood while sampling the aortic blood, we have demonstrated in a previous study that small fragments of brain tissue are capable of passing through the fine lung capillaries (Coore et al., 2004[b]). The subsequent considerable dilution in the blood of this brain material necessitates the use of a highly sensitive detection method to identify any contamination with this material in visceral organs. The PCR assay applied in this experiment proved to be highly sensitive with a detection limit of 0.05%, determined by analysis of dilutions of known amounts of male DNA in buffer, a figure that compares favourably with PCR assays used in related studies (Bottero et al., 2003). This detection limit is equivalent to 0.5-µg of brain material per 5-µl of buffer (100-µg/ml). This detection limit compares with the detection limits determined during the validation of the ELISA and analysis by microscopy and immunocytochemistry.

Although the detection limit of the PCR is not substantially better than that achieved by the GFAP ELISA (refer to 3.1.3.9.1.1 & 3.1.3.9.2.1), it does offer advantages for the detection of brain tissues in organs. High background levels of GFAP in organs prevented detection of low levels of contamination by ELISA leading to a reduction in the specificity of the assay (refer to 3.1.3.8.1). Since the SRY (sex-determining region of the Y-chromosome) DNA is present only in the Y- chromosome there is no possibility for cross-reaction from other sources, giving the potential for high specificity of the PCR. An explanation for the level of sensitivity achieved by the PCR may be that the SRY chromosomal DNA is a single copy gene present only once in each cell. This would mean that at low concentrations of male brain tissue contamination in organs there may be insufficient DNA present to be amplified enough for detection to be possible.

The detection of the SRY DNA in lymphatic tissues from a female sheep conducted as part of the validation of the PCR assay indicated that the assay could detect small quantities of tissue contamination. The failure to detect any SRY DNA in the organs of

a sheep following injection with brain suspension into the jugular venous blood, suggests that if any contamination occurred, it was below the limit of detection of this sensitive assay. An alternative explanation may rest with a draw-back of the assay which still relies on sampling of minute quantities of the organ for analysis. Incomplete homogenisation and mixing of the organ specimens may have resulted in pockets of male brain tissue in organs that were simply not taken for DNA extraction and analysis. To reduce the risk of this in future experiments using this method, complete digestion of an entire organ with large volumes of the enzyme Proteinase K followed by DNA extraction of the tissue digest may ensure that any male tissue contamination was not over-looked.

Previous studies investigating organ contamination using a bacterial marker found extensive contamination of muscles and of organs with the marker (Buncic et al., 2002; Prendergast et al., 2004). The application of such studies to investigations of the dissemination of brain tissue in the carcass is open to question since the morphology and dynamics of bacteria and brain tissue fragments in blood may be very different. In addition, the use of a replicating marker while useful for demonstrating potential contamination does not allow quantification of the brain tissue contamination that could occur.

It is possible that low levels of organ contamination may be detected using this technique of male brain tissue injection and subsequent DNA extraction from tissues and PCR analysis if further studies are conducted, using larger sample populations of animals.

## 4.6 INVESTIGATION OF PROTHROMBIN TIME ESTIMATION OF OVINE PLASMA AS AN INDIRECT TEST FOR BRAIN TISSUE EMBOLI IN BLOOD

This experiment attempts to identify the presence of brain tissue fragments in jugular blood samples taken after CBG stunning by measurement of prothrombin time in the plasma. By estimation of the amount of thromboplastin released into the blood samples it may be possible to separate samples in which brain tissue fragments are present. The more thromboplastin present in a sample the more clotting should occur with the accompanying exhaustion of more clotting factors. This should cause prothrombin

times to be prolonged in these samples when compared to samples in which less thromboplastin was present and in fact this is what was observed in the series of sheep plasma samples examined. Comparison of the mean pre-stun plasma PT times with that of the mean post-stun plasma PT times after use of both types of captive bolt gun shows a very significant difference in the time for clotting to occur. The post-stun sample PT times were considerably longer than those observed from the pre-stun plasma samples. The explanation for this observed difference lies with the release of thromboplastin from damaged tissue as a result of CBG stunning. Although thromboplastin is found in high concentrations in brain tissue it is also found in all other tissues albeit in smaller quantities. The ubiquitous nature of this potent activator of the extrinsic clotting pathway suggests that it may not be ideal as a marker solely for brain tissue. Nevertheless, while it was expected that all post-stun samples might demonstrate prolonged PT times over the pre-stun samples it was hoped that samples containing brain tissue fragments might show PT times prolonged even beyond these times. This may in fact have occurred in samples containing brain tissue but may be indistinguishable from other post-stun samples since most post-stun samples demonstrate PT times beyond 70-seconds which are the limit of recordable PT times on the coagulation analyser used in the study. Consequently, no separation is possible among samples in which PT times are beyond 70-seconds. In future studies on this subject it may be worthwhile either manually timing PT times to get an accurate reading from all samples including those with prolonged PT times or by using an alternative automated system that allows a greater range of PT time recording.

The sheep plasma samples produced consistent elevation of PT times within sixty seconds of stunning and in fact comparison of mean pre-stun PT times with the PT time of samples taken within ten seconds of stunning also produces a significant difference ($P<0.05$). The rapidity of the clotting process after severe head trauma is not previously described although prolonged PT times have been observed after analysis of plasma obtained from victims of head trauma at various intervals post injury (Kuo, Chou & Chio, 2003). The advantage offered by this study is that sampling began almost immediately post-stunning and continued over one minute.

Investigation of PT times within the first minute of head trauma may provide useful information for research on the activation of the clotting cascade after head trauma or even upon that which might occur during surgery on the brain. The results of this study

indicate that the clotting cascade is activated almost simultaneously with the application of head trauma.

## 4.7 INVESTIGATION OF PROTHROMBIN TIME ESTIMATION OF BOVINE PLASMA AS AN INDIRECT TEST FOR BRAIN TISSUE EMBOLI IN BLOOD

Analysis of the prothrombin times obtained from assay of bovine plasma indicated a significant difference between the pre and post-stunning samples from animals in which brain tissue embolism had occurred. No significant difference was demonstrated between pre-and post stun samples prothrombin times from negative samples. This suggests that estimation of prothrombin time in bovine plasma may offer a method of distinguishing samples taken from animals with brain tissue emboli after stunning. While statistical analysis did establish significance of this result, the small sample size of the positive group of animals must reduce the confidence in this association. A larger sample group of cattle with brain tissue emboli after stunning would be necessary to confirm these results.

An explanation of the success of the assay as a tool for identifying bovine samples containing emboli although not from ovine samples may stem from the much larger volume of blood in cattle as compared sheep. After captive bolt gun stunning similar levels of head and brain trauma occur in both species and it might be expected that comparable amounts of thromboplastin would be released into the circulation. In cattle, the dilution effects of the large blood volume on the thromboplastin may lead to a failure of activation of the clotting cascade in most cases. Only in instances in which brain tissue emboli are also present is a sufficiently high concentration achieved to activate the clotting cascade.

In sheep, with a much smaller blood volume as compared to cattle, the comparatively low concentrations of thromboplastin released from head and brain tissue trauma may be sufficient to activate the clotting cascade in all cases. This may explain the difference observed in the results from analysis of the plasma samples from each species.

Further studies upon the effects of brain tissue emboli on coagulation might examine a range of parameters of coagulation including D-dimer concentrations, activated partial thromboplastin time (APTT), thrombin clotting time, fibrinogen assay, and fibrinogen degradation products. The use of these additional measures of coagulation may increase the sensitivity of the method for brain tissue emboli detection.

## 4.8 IMPROVED DESIGNS OF CAPTIVE BOLT GUN

While this study has involved the investigation of a range of issues and factors relating to the use of captive bolt guns and the occurrence of brain tissue emboli, the desired outcome of these studies must be a reduction of the risk posed by the use of these stunning methods.

Captive bolt guns have been used in the humane stunning and slaughter of livestock for over one hundred years with only minor evolution in design. An ideal stunning technique should fulfil these requirements:

1) Produce an immediate and reproducible stun for maintenance of good welfare standards
2) Must be economic and widely accessible and acceptable (religious slaughter) through-out the world
3) Ease of operation and safety for the operator
4) Does not encourage embolism of brain tissue into the venous return

The last of these requirements is recent and has only become important since the BSE outbreak in cattle and the discovery that captive bolt gun stunning can sometimes cause brain tissue emboli to enter the circulation (Anil et al. 1999; 2001; 2002). The results of this study suggest the need for the development of a humane stunning method that avoids the risk of brain tissue embolism. The economic realities of the beef industry combined with the recorded problems with the currently available use of electrical stunning on cattle indicate that electrical stunning may not be the only answer. Many of the current problems associated with electrical stunning methods used on cattle relate to welfare and to meat quality issues. Application of the electrodes may be associated with a brief period of pain before unconsciousness is induced, particularly if poor contact occurs between the electrodes and the skin. Furthermore, electrical stunning

may in some cases lead to signs of returning consciousness before sticking, or to failure to initiate cardiac arrest leading to the necessity of repeat stunning by re-application of the electrodes or by captive bolt gun stunning. Meat quality may be adversely affected after use of electrical stunning by way of muscle haemorrhages and by the occurrence of bone fractures. The high cost of electrical stunning equipment for cattle is a further obstacle for the use of this method especially in smaller abattoirs with modest through-put of livestock.

Modifications of current bolt designs have previously been suggested many of which have relied upon reducing the damage inflicted on the brain by the bolt. At a recent meeting initiated by the European Commission and attended by academics and representatives from the industry, a variety of novel and modified stunning methods were discussed. Novel stunning methods discussed at the meeting included, the potential use of trans-cranial magnetic stimulation (TMS) and improvements to electrical stunning methods currently in use. The main outputs from this seminar were later included in a report to the European Commission (Safestun, accompanying measures QLK2-2002-3051).

The wide breath of this study encompassing both trials with CBGs and anatomical studies has provided in-sights into possible mechanisms by which embolism occurs and also has led to theoretical suggestions for CBG modifications. The necessity for modifications to current CBG designs has been suggested for other reasons besides that of brain tissue embolisation. During the recent Foot and Mouth disease epidemic in the UK the unprecedented call for wide-scale slaughter of livestock as a method of controlling the spread of disease led to a welfare disaster. The need to slaughter a large number of animals rapidly resulted in the use of captive bolt guns as a method of slaughter causing some animals to exhibit signs of recovery in the cull. In the period since the cull there has been a call by the UK government for a new design of CBG gun that will kill as well as induce a stun.

Recent studies have revealed information on the drainage of emboli from the head (Coore et al. 2004[b]) and also on the dissemination of emboli within the carcass including entry into the aortic blood supply (Coore et al. 2004[a]). The mechanism of entry of brain tissue emboli into the venous circulation after stunning remains unclear. The dorsal sagittal sinus has been suggested as an entry point for emboli following gun

shot to head and raised intra-cranial pressure has been sited as a major pre-disposing factor (Miyaishi et al., 1994).

Anatomical studies that we have completed using a technique of resin corrosion casts of the cerebral venous drainage in sheep and cattle suggests an alternative entry point for brain tissue fragments after CBG stunning. The casts demonstrate that while the dorsal sagittal sinus will undoubtedly be ruptured by the passage of the bolt, the vessel itself is of modest proportions, even in an adult cow. Measurements of the vessel obtained from the prepared cast of the adult bovine, suggest a diameter of only 8-mm at its widest point and only 2-mm in diameter for much of its length. On the other hand, the basilar system of veins located on the ventral surface of the brain is much more extensive and of greater proportions of up to 35-mm in diameter. Estimations based on the average length of the bolt and the depth of the cranial cavity indicates that the basilar vessels will also be ruptured by the passage of the bolt in many instances. This statement is supported by examination of cattle and sheep brains following CBG stunning (Farag, 2002; Biggins, 2003). It appears much more likely that it is this ventrally situated plexus of vessels that is the entry point for brain tissue emboli, rather than the dorsal sagittal sinus.

Based on this hypothesis it would seem sensible to modify current designs of captive bolt gun to reduce the risk of rupture of these ventrally situated vessels. Although vessels may be ruptured by concussive force alone, the length of the bolt of a penetrating gun ensures that these vessels will always be traumatised, at least in sheep, where the bolt strikes the base of the cranial cavity.

Entry of projectiles including captive bolts into the skull is known to result in raised intra-cranial pressure, as a result of several contributing factors (Blackmore, 1985). Such factors may include the following:

1) Entry of gas into the cranium generated by the explosive discharge of the projectile
2) Formation of a temporary cavity (cavitation effect)
3) Displacement of tissue by the volume of the bolt (~12.5cm$^3$) [$2\pi r^2 h$ where r=0.5cm and h=8cm]

Cavitation refers to the temporary cavity created by radial forces imparted to the brain parenchyma by the passage of the bolt. It persists for 5 to 30-microseconds and may have a diameter of 30 to 40 times that of the projectile and generate local internal pressures greater than 10-mPa within the expanding walls of the cavity (Finnie, 2000). Displacement of tissues by the space-occupying bolt will further raise this pressure presumably only for the microseconds that the bolt remains in the skull before it returns to the barrel. Evidence for this rapid increase in intra-cranial pressure has been demonstrated by experiments in which de-capitated heads were shot with the captive bolt gun and was followed by an explosive discharge of brain material from the foramen magnum (Finnie, 2000).

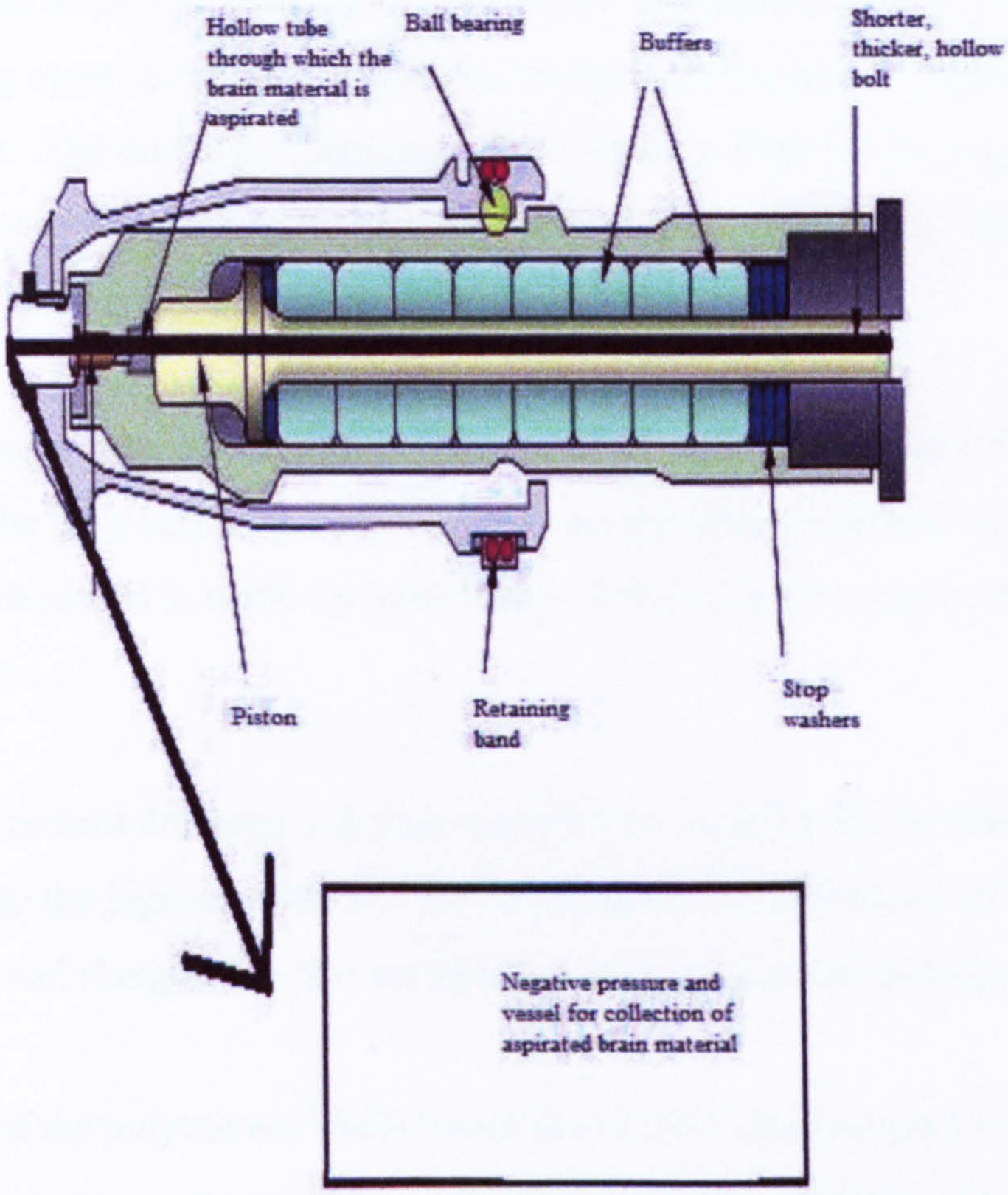
The increased intra-cranial pressure produced by the entry of the bolt into the skull provides the best explanation for why brain tissue fragments sometimes enter the venous sinuses after stunning. While the venous system is a low-pressure environment, one would expect that it should still be higher than that present in the surrounding tissues under normal circumstances. Emboli are very unlikely to passively drift into an area of positive pressure from the surrounding brain tissue, if the pressures within the cranial cavity were lower than those in the venous system. It seems probable that emboli are forced into the venous sinuses by a brief but significant rise in intra-cranial pressure. Following extraction of the bolt, the system is no longer a closed one and intra-cranial pressures should fall. The extrusion of brain material from the bolt-hole is a result of the equalising pressures between the external environment and within the skull. This explanation of the pressure as the main factor rather than brain fragmentation and haemorrhage is born out by the recent prevalence studies of brain tissue embolism in cattle and sheep subjects (Coore et al., 2004; 2005- in press). The significantly higher prevalence obtained in the sheep study over that of the cattle study can be explained by the higher intra-cranial pressures expected within the smaller sheep cranium than in the bovine cranial cavity. Recent studies involving the macroscopic examination of both sheep and cattle brains after captive bolt stunning have failed to produce evidence of either greater haemorrhage or generally greater quantities of loose brain in sheep than in cattle (Farag, 2002; Biggins, 2003). It is likely that in sheep, the high intra-cranial pressures force emboli into the venous sinuses more frequently than in the bovine. The bolts used in both cases are of similar volumes, albeit discharged with greater force in cattle.

Based on the findings from previous work and from the proceeding explanation a modification of captive bolt design is suggested that may reduce the problem of brain tissue embolism while maintaining the other requirements of a good mechanical stunning device. The basis for this suggestion is the provision for an alternative outlet for the brain tissue that might otherwise become an embolus in the venous return. To be effective this other exit would need to be created simultaneously with the bolts penetration of the skull and be sufficiently large to reduce internal cranial pressure. The downside of this approach might be to reduce the effectiveness of the stun, since raised intra-cranial pressure is known to cause tearing and stretching of axons that contribute to the effectiveness of the stun and reduce the chances of a return to consciousness.

### 4.8.1 Modifications to a penetrating captive bolt gun

In this modification to the gun, a hollow bolt is created through which brain tissue under pressure might exit. Connecting this system to a suction device might increase the efficiency and reduce blockage of the outlet with loose tissue. It might also serve to collect the brain tissue obtained and prevent contamination of the abattoir environment. In addition, the bolt is shortened to approximately 6-cm while the diameter of the bolt is increased to maintain the durability of the hollow bolt.

Figure 4.1 Modified penetrating captive bolt gun providing an outlet for brain material to exit the cranium while the shorter bolt length should reduce trauma to the cerebral sinuses lying on the ventral aspect of the bovine brain



## 4.9 CONCLUSIONS

The use of captive bolt gun stunning of cattle and sheep has been demonstrated to cause brain tissue emboli to enter the venous return at frequencies of from 2 to 23% depending upon the species of stunned animal and the type of captive bolt gun employed. The amount of brain tissue that may be found in the jugular vein blood of sheep after captive bolt gun stunning was found in a small pilot study to have a mean weight of 0.97-g (range 0.22 to 2.08).

The potential of small fragments of brain tissue up to a diameter of 20-μm to pass through the lung capillaries of sheep to enter the arterial circulation has been demonstrated and in cattle for brain tissue proteins to enter the arterial circulation by the same route.

Venous cerebral drainage was demonstrated to occur by the vertebral system of veins in addition to the jugular veins and the potential for dissemination of brain tissue emboli at stunning and slaughter by the vertebral venous system was examined.

The use of the polymerase chain reaction to detect small amounts of marker DNA in a carcass was demonstrated to be a sensitive and practical technique of investigating the potential for brain tissue contamination of carcass organs as a result of stunning.

The use of a coagulation test was shown to be a potential method of identifying plasma samples from cattle in which brain tissue had entered the circulation after stunning. Analysis of sheep plasma samples, indicated that the coagulation test was a sensitive method of detecting brain trauma within 10-s of the injury including that which resulted in brain tissue emboli.

The information gained from these studies has been applied towards possible modifications to current captive bolt gun design aimed at reducing the risk of brain tissue emboli after captive bolt gun stunning.

## 4.10 RECOMMENDATIONS

- Research on novel stunning methods as well as modified versions of current methods

- Further studies on the potential for dissemination of brain tissue emboli in the carcass conducted under commercial conditions

- Development of improved analytic methods for the detection and quantification of stunning induced brain tissue contamination of the carcass

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# APPENDICES

# APPENDIX 1 RAW DATA FROM THE VALIDATION OF THE GFAP ELISA ON CATTLE AND SHEEP SAMPLES

# APPENDIX 1 RAW DATA FROM THE VALIDATION OF THE GFAP ELISA ON CATTLE AND SHEEP SAMPLES

## Data obtained using bovine tissue samples

Table 1A OD values for the dilution curve of bovine brain tissue in blood

| Concentration | Dose dilution curve (OD Value) | | | | | | |
|---|---|---|---|---|---|---|---|
| (mg/ml) | 1 | 2 | 3 | 4 | 5 | Mean | SD |
| 10.00 | 3.07 | 2.95 | 2.86 | 3.15 | 2.90 | 2.99 | 0.12 |
| 5.00 | 3.04 | 3.04 | 2.92 | 3.08 | 2.85 | 2.99 | 0.10 |
| 2.50 | 3.14 | 2.79 | 2.86 | 3.11 | 2.88 | 2.96 | 0.16 |
| 1.25 | 2.40 | 2.56 | 2.85 | 3.04 | 2.85 | 2.74 | 0.26 |
| 0.6250 | 1.43 | 2.88 | 2.89 | 1.18 | 2.17 | 2.11 | 0.80 |
| 0.3125 | 0.87 | 0.78 | 2.03 | 0.90 | 1.51 | 1.22 | 0.54 |
| 0.1560 | 0.47 | 0.89 | 1.51 | 0.37 | 0.46 | 0.74 | 0.48 |
| 0.0780 | 0.28 | 0.34 | 0.77 | 0.25 | 0.21 | 0.37 | 0.23 |
| 0.0390 | 0.17 | 0.14 | 0.41 | 0.14 | 0.16 | 0.20 | 0.11 |
| 0.0195 | 0.11 | 0.09 | 0.16 | 0.16 | 0.15 | 0.13 | 0.03 |
| 0.0098 | 0.09 | 0.08 | 0.11 | 0.07 | 0.11 | 0.09 | 0.02 |
| 0.0049 | 0.06 | 0.05 | 0.06 | 0.06 | 0.05 | 0.06 | 0.01 |
| 0.0024 | 0.07 | 0.07 | 0.06 | 0.06 | 0.06 | 0.06 | 0.01 |
| 0.0012 | 0.04 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 | 0.01 |
| 0.0006 | 0.07 | 0.04 | 0.04 | 0.04 | 0.09 | 0.06 | 0.02 |

Table2A. Detectable concentrations of GFAP in bovine brain in blood dilutions (Dilution 1)

| Sample number | Brain Concentration mg/ml | OD Value | GFAP ng/ml |
|---|---|---|---|
| 1 | 10.0000 | 3.070 | S |
| 2 | 5.0000 | 3.041 | S |
| 3 | 2.5000 | 3.140 | S |
| 4 | 1.2500 | 2.400 | 13.78 |
| 5 | 0.6250 | 1.430 | 6.34 |
| 6 | 0.3125 | 0.866 | 3.05 |
| 7 | 0.1560 | 0.466 | 1.64 |
| 8 | 0.0780 | 0.281 | 0.775 |
| 9 | 0.0390 | 0.169 | nd |
| 10 | 0.0195 | 0.105 | nd |
| 11 | 0.0098 | 0.087 | nd |
| 12 | 0.0049 | 0.056 | nd |
| 13 | 0.0024 | 0.069 | nd |
| 14 | 0.0012 | 0.043 | nd |
| 15 | 0.0006 | 0.065 | nd |

S- Saturated levels

nd-Not detectable

Table 3A Detectable concentrations of GFAP in bovine brain in blood dilutions (Dilution 2)

| Sample number | Brain Concentration mg/ml | OD Value | GFAP ng/ml |
|---|---|---|---|
| 16 | 10.0000 | 2.950 | S |
| 17 | 5.0000 | 3.040 | S |
| 18 | 2.5000 | 2.790 | S |
| 19 | 1.2500 | 2.560 | S |
| 20 | 0.6250 | 2.880 | S |
| 21 | 0.3125 | 0.780 | S |
| 22 | 0.1560 | 0.891 | 3.3 |
| 23 | 0.0780 | 0.342 | 1.05 |
| 24 | 0.0390 | 0.144 | nd |
| 25 | 0.0195 | 0.091 | nd |
| 26 | 0.0098 | 0.079 | nd |

| | | | |
|---|---|---|---|
| 27 | 0.0049 | 0.054 | nd |
| 28 | 0.0024 | 0.073 | nd |
| 29 | 0.0012 | 0.059 | nd |
| 30 | 0.0006 | 0.043 | nd |

S- Saturated levels

nd-Not detectable

Table 4A Detectable concentrations of GFAP in bovine brain in blood dilutions (Dilution 3)

| Sample Number | Brain Concentration mg/ml | OD Value | GFAP ng/ml |
|---|---|---|---|
| 46 | 10.0000 | 2.860 | S |
| 47 | 5.0000 | 2.920 | S |
| 48 | 2.5000 | 2.860 | S |
| 49 | 1.2500 | 2.850 | S |
| 50 | 0.6250 | 2.890 | S |
| 51 | 0.3125 | 2.030 | 11.08 |
| 52 | 0.1560 | 1.510 | 7.42 |
| 53 | 0.0780 | 0.772 | 3.01 |
| 54 | 0.0390 | 0.405 | 1.53 |
| 55 | 0.0195 | 0.161 | nd |
| 56 | 0.0098 | 0.114 | nd |
| 57 | 0.0049 | 0.064 | nd |
| 58 | 0.0024 | 0.062 | nd |
| 59 | 0.0012 | 0.046 | nd |
| 60 | 0.0006 | 0.040 | nd |

S- Saturated levels

nd-Not detectable

Table 5A Detectable concentrations of *GFAP in bovine brain in blood dilutions*

(Dilution 4)

| Sample number | Brain Concentration mg/ml | OD Value | GFAP ng/ml |
|---|---|---|---|
| 61 | 10.0000 | 3.150 | S |
| 62 | 5.0000 | 3.080 | S |
| 63 | 2.5000 | 3.112 | S |
| 64 | 1.2500 | 3.040 | S |
| 65 | 0.6250 | 1.180 | 5.24 |
| 66 | 0.3125 | 0.895 | 2.432 |
| 67 | 0.1560 | 0.374 | 0.877 |
| 68 | 0.0780 | 0.247 | 0.58 |
| 69 | 0.0390 | 0.137 | nd |
| 70 | 0.0195 | 0.159 | nd |
| 71 | 0.0098 | 0.073 | nd |
| 72 | 0.0049 | 0.059 | nd |
| 73 | 0.0024 | 0.057 | nd |
| 74 | 0.0012 | 0.045 | nd |
| 75 | 0.0006 | 0.044 | nd |

S- Saturated levels

nd-Not detectable

Table 6A Detectable concentrations of GFAP in bovine brain in blood dilutions

(Dilution 5)

| Sample number | Brain Concentration mg/ml | OD Value | GFAP ng/ml |
|---|---|---|---|
| 76 | 10.0000 | 2.900 | S |
| 77 | 5.0000 | 2.850 | S |
| 78 | 2.5000 | 2.880 | S |
| 79 | 1.2500 | 2.850 | S |
| 80 | 0.6250 | 2.170 | 9.75 |
| 81 | 0.3125 | 1.510 | 5.21 |
| 82 | 0.1560 | 0.456 | 1.06 |
| 83 | 0.0780 | 0.208 | 0.497 |
| 84 | 0.0390 | 0.162 | nd |
| 85 | 0.0195 | 0.150 | nd |
| 86 | 0.0098 | 0.112 | nd |

| | | | |
|---|---|---|---|
| 87 | 0.0049 | 0.045 | nd |
| 88 | 0.0024 | 0.062 | nd |
| 89 | 0.0012 | 0.048 | nd |
| 90 | 0.0006 | 0.086 | nd |

S- Saturated levels

nd-Not detectable

Table 7A OD values for the standard curve of the GFAP ELISA

| Concentration | GFAP standard curve (OD Value) | | | | | | |
|---|---|---|---|---|---|---|---|
| (ng/ml) | 1 | 2 | 3 | 4 | 5 | Mean | SD |
| 2000 | 3.00 | 3.00 | 3.15 | 2.81 | 3.01 | 3.00 | 0.121 |
| 1000 | 2.88 | 2.93 | 2.97 | 2.72 | 2.74 | 2.85 | 0.110 |
| 500 | 2.95 | 2.95 | 2.95 | 2.71 | 2.87 | 2.89 | 0.101 |
| 250 | 2.98 | 2.81 | 2.87 | 2.80 | 2.81 | 2.86 | 0.076 |
| 125 | 3.19 | 2.94 | 2.93 | 2.80 | 2.71 | 2.91 | 0.183 |
| 62.5 | 2.99 | 2.96 | 2.97 | 2.81 | 2.77 | 2.90 | 0.101 |
| 31.25 | 2.99 | 3.08 | 3.04 | 2.73 | 2.74 | 2.92 | 0.167 |
| 15.625 | 3.26 | 3.32 | 3.28 | 2.74 | 2.94 | 3.11 | 0.258 |
| 7.812 | 2.69 | 2.67 | 2.91 | 1.26 | 2.38 | 2.38 | 0.652 |
| 3.906 | 1.66 | 1.57 | 1.97 | 0.56 | 0.86 | 1.32 | 0.592 |
| 1.976 | 1.03 | 1.02 | 1.35 | 0.36 | 0.60 | 0.87 | 0.390 |
| 0.976 | 0.52 | 0.49 | 0.75 | 0.20 | 0.31 | 0.45 | 0.210 |
| 0.488 | 0.34 | 0.30 | 0.43 | 0.11 | 0.18 | 0.27 | 0.127 |
| 0.288 | 0.20 | 0.17 | 0.20 | 0.08 | 0.13 | 0.16 | 0.051 |
| 0.122 | 0.09 | 0.10 | 0.11 | 0.06 | 0.08 | 0.09 | 0.021 |

**Data obtained using ovine tissue samples**

Table 8A OD values for the dilution curve of ovine brain tissue in blood

| Concentration | Dose dilution curve (OD Value) | | | | | | |
|---|---|---|---|---|---|---|---|
| (mg/ml) | 1 | 2 | 3 | 4 | 5 | Mean | SD |
| 10.0000 | 2.83 | 2.98 | 3.10 | 3.10 | 2.97 | 3.00 | 0.11 |
| 5.0000 | 2.85 | 2.81 | 3.08 | 3.06 | 2.80 | 2.92 | 0.14 |
| 2.5000 | 2.97 | 2.35 | 1.26 | 2.34 | | 2.23 | 0.71 |
| 1.2500 | 3.06 | 1.85 | 2.13 | 1.30 | 0.82 | 1.83 | 0.85 |
| 0.6250 | 1.42 | 0.78 | 0.98 | 0.93 | 0.43 | 0.91 | 0.36 |
| 0.3130 | 1.13 | 0.58 | 0.39 | 0.36 | 0.29 | 0.55 | 0.34 |
| 0.1560 | 0.96 | 0.21 | 0.24 | 0.18 | 0.24 | 0.36 | 0.33 |
| 0.0780 | 0.70 | 0.11 | 0.32 | 0.14 | 0.23 | 0.30 | 0.24 |
| 0.0390 | 0.08 | 0.09 | 0.12 | 0.13 | 0.15 | 0.11 | 0.03 |
| 0.0195 | 0.09 | 0.06 | 0.08 | 0.12 | 0.54 | 0.18 | 0.20 |
| 0.0098 | 0.08 | 0.06 | 0.09 | 0.12 | 0.11 | 0.09 | 0.02 |
| 0.0049 | 0.13 | 0.04 | 0.10 | 0.12 | 0.12 | 0.10 | 0.04 |
| 0.0024 | 0.05 | 0.04 | 0.10 | 0.12 | 0.25 | 0.11 | 0.08 |
| 0.0012 | 0.07 | 0.05 | 0.08 | 0.04 | 0.25 | 0.10 | 0.09 |
| 0.0006 | 0.04 | 0.04 | 0.09 | 0.03 | 0.21 | 0.08 | 0.08 |

Table 9A Detectable concentrations of GFAP in ovine brain in blood dilutions (Dilution1)

| Sample number | Brain concentrations mg/ml | OD value | GFAP ng/ml |
|---|---|---|---|
| 16 | 10.000 | 2.7 | S |
| 17 | 5.000 | 2.4 | S |
| 18 | 2.500 | 2.6 | S |
| 19 | 1.25 | 1.5 | 7.8 |
| 20 | 0.6250 | 1.5 | 7.7 |
| 21 | 0.3125 | 0.32 | 1.46 |
| 22 | 0.1560 | 0.787 | 4.2 |
| 23 | 0.078 | 0.093 | nd |
| 24 | 0.0390 | 0.014 | nd |
| 25 | 0.0195 | 0.007 | nd |
| 26 | 0.0098 | 0.003 | nd |
| 27 | 0.0049 | 0.205 | nd |
| 28 | 0.0024 | 0.005 | nd |
| 29 | 0.0012 | 0.018 | nd |
| 30 | 0.0006 | 0.012 | nd |

S-Saturated levels
nd-Not detectable

Table 10A Detectable concentrations of GFAP in ovine brain in blood dilutions (Dilution 2)

| Sample number | Brain concentrations mg/ml | OD value | GFAP ng/ml |
|---|---|---|---|
| 31 | 10.000 | 3.058 | S |
| 32 | 5.000 | 2.143 | S |
| 33 | 2.500 | 1.57 | 7.6 |
| 34 | 1.25 | 0.873 | 4.4 |
| 35 | 0.6250 | 0.185 | 0.83 |
| 36 | 0.3125 | 0.14 | nd |
| 37 | 0.1560 | 0.05 | nd |
| 38 | 0.078 | 0.06 | nd |
| 39 | 0.0390 | 0.007 | nd |
| 40 | 0.0195 | 0.042 | nd |
| 41 | 0.0098 | 0.003 | nd |
| 42 | 0.0049 | 0.007 | nd |
| 43 | 0.0024 | 0.006 | nd |
| 44 | 0.0012 | 0.085 | nd |
| 45 | 0.0006 | 0.004 | nd |

S-Saturated levels
nd-Not detectable

Table 11A Detectable concentrations of GFAP in ovine brain in blood dilutions (Dilution 3)

| Sample number | Brain concentrations mg/ml | OD value | GFAP ng/ml |
|---|---|---|---|
| 46 | 10.000 | 2.79 | S |
| 47 | 5.000 | 2.74 | S |
| 48 | 2.500 | 2.84 | S |
| 49 | 1.25 | 1.78 | 9.6 |
| 50 | 0.6250 | 0.88 | 4.6 |
| 51 | 0.3125 | 0.64 | 3.3 |
| 52 | 0.1560 | 0.32 | 1.4 |
| 53 | 0.078 | 0.18 | 0.8 |
| 54 | 0.0390 | 0.091 | nd |
| 55 | 0.0195 | 0.053 | nd |
| 56 | 0.0098 | 0.078 | nd |
| 57 | 0.0049 | 0.02 | nd |
| 58 | 0.0024 | 0.05 | nd |
| 59 | 0.0012 | 0.08 | nd |
| 60 | 0.0006 | 0.03 | nd |

S-Saturated levels
nd-Not detectable

Table 12A Detectable concentrations of GFAP in ovine brain in blood dilutions (Dilution 4)

| Sample number | Brain concentrations mg/ml | OD value | GFAP ng/ml |
|---|---|---|---|
| 61 | 10.000 | 3.01 | S |
| 62 | 5.000 | 1.74 | 7.5 |
| 63 | 2.500 | 0.69 | 5 |
| 64 | 1.25 | 0.72 | 5.2 |
| 65 | 0.6250 | 0.27 | 1.8 |
| 66 | 0.3125 | 0.20 | 1.4 |
| 67 | 0.1560 | 0.39 | 2.9 |
| 68 | 0.078 | 0.058 | nd |
| 69 | 0.0390 | 0.31 | 2.0 |
| 70 | 0.0195 | 0.31 | 2.0 |
| 71 | 0.0098 | 0.02 | nd |
| 72 | 0.0049 | 0.07 | nd |
| 73 | 0.0024 | 0.16 | nd |
| 74 | 0.0012 | 0.08 | nd |
| 75 | 0.0006 | 0.086 | nd |

S-Saturated levels
nd-Not detectable

Table 13A Detectable concentrations of GFAP in ovine brain in blood dilutions (Dilution 5)

| Sample number | Brain concentrations mg/ml | OD value | GFAP ng/ml |
|---|---|---|---|
| 76 | 10.000 | 2.8 | S |
| 77 | 5.000 | 2.723 | S |
| 78 | 2.500 | 2.735 | S |
| 79 | 1.25 | 2.0 | 16.9 |
| 80 | 0.6250 | 2.27 | 21.3 |
| 81 | 0.3125 | 1.08 | 7.5 |
| 82 | 0.1560 | 0.084 | nd |
| 83 | 0.078 | 0.086 | nd |
| 84 | 0.0390 | 0.06 | nd |
| 85 | 0.0195 | 0.135 | nd |
| 86 | 0.0098 | 0.13 | nd |
| 87 | 0.0049 | 0.093 | nd |
| 88 | 0.0024 | 0.124 | nd |
| 89 | 0.0012 | N/A | N/A |
| 90 | 0.0006 | N/A | N/A |

S-Saturated levels
nd-Not detectable

N/A -Not available

Table 14A OD values obtained from the dilutions of the GFAP standard

| Concentration | GFAP standard curve (OD Value) | | | | | | |
|---|---|---|---|---|---|---|---|
| (ng/ml) | 1 | 2 | 3 | 4 | 5 | Mean | SD |
| 2000 | 3.02 | 3.12 | 3.27 | 2.99 | 3.18 | 3.12 | 0.12 |
| 1000 | 2.62 | 2.97 | 2.89 | 2.81 | 2.94 | 2.85 | 0.14 |
| 500 | 2.98 | 3.13 | 2.98 | 2.91 | 2.97 | 2.99 | 0.08 |
| 250 | 2.95 | 2.99 | 2.98 | 2.81 | 2.88 | 2.92 | 0.08 |
| 125 | 3.14 | 3.07 | 3.03 | 2.83 | 2.96 | 3.01 | 0.12 |
| 62.5 | 2.67 | 3.05 | 3.03 | 2.96 | 2.88 | 2.92 | 0.16 |
| 31.25 | 2.95 | 3.29 | 3.02 | 3.08 | 2.57 | 2.98 | 0.26 |
| 15.625 | 2.91 | 3.17 | 3.04 | 2.55 | 1.36 | 2.60 | 0.73 |
| 7.812 | 1.09 | 1.94 | 1.76 | 1.39 | 0.60 | 1.36 | 0.54 |
| 3.906 | 0.38 | 0.93 | 1.26 | 0.39 | 0.37 | 0.66 | 0.41 |
| 1.953 | 0.27 | 0.67 | 0.68 | 0.34 | 0.16 | 0.42 | 0.24 |
| 0.976 | 0.14 | 0.37 | 0.40 | 0.29 | 0.11 | 0.26 | 0.13 |
| 0.488 | 0.11 | 0.18 | 0.16 | 0.14 | 0.08 | 0.13 | 0.04 |
| 0.244 | 0.07 | 0.11 | 0.12 | 0.08 | 0.06 | 0.09 | 0.03 |
| 0.122 | 0.04 | 0.06 | 0.07 | 0.06 | 0.05 | 0.06 | 0.01 |

# APPENDIX 2 PUBLISHED PAPERS AND ABSTRACTS

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## Papers

R.R. Coore, S. Love, J.L. Mckinstry, H.R. Weaver, A. Phillips, T. Hillman, M.J.Hiles, A. Shand, C.R. Helps and M.H. Anil (2004). Dissemination of brain emboli following captive bolt stunning of sheep: capacity for entry into the systemic arterial circulation. *Journal of Food Protection,* **67**, 1050-1052

R.R. Coore, F.J. Barr, J.L. Mckinstry, M.H. Anil. (2004). Neural embolism and cerebral venous drainage at stunning and slaughter. *Veterinary Record,* **155**, 86-87

R.R. Coore, S. Love, C.R. Helps and M.H. Anil. (2004). Frequency of brain tissue embolism associated with captive bolt gun stunning of sheep. *Foodborne Pathogens and Disease,* **1**, 291-294

R.R. Coore, S. Love, J.L. McKinstry, H.R. Weaver, A. Philips, T. Hillman, M. Hiles, C.R. Helps and M.H. Anil. (2005) Brain tissue fragments in jugular-vein blood of cattle stunned by use of penetrating or non-penetrating captive bolt guns. *Journal of Food Protection,* **68**, 882-884

## Abstracts

R.R. Coore, S. Love, J.L. Mckinstry, H.R. Weaver, A. Philips, T. Hillman, C.R. Helps and M.H.Anil.(2003). The prevalence and potential for dissemination of neural embolism in cattle and sheep following the use of mechanical stunning methods currently used in the UK 49th International Congress of Meat Science and Technology (ICOMST), September 2003, Brazil

R.R. Coore, S. Love & M.H. Anil. (2004). The frequency and capacity for dissemination of brain tissue embolism associated with pre-slaughter stunning of cattle. Joint Funders Transmissible Spongiform Encephalopathies Workshop, 1-3 September 2004, University of York, UK

R.R. Coore, S. Love & M.H. Anil. (2004). The frequency and capacity for dissemination of brain tissue embolism associated with pre-slaughter stunning of sheep. Joint Funders Transmissible Spongiform Encephalopathies Workshop, 1-3 September 2004, University of York, UK

R.R. Coore, S. Love & M.H. Anil (2004). The frequency and capacity for dissemination of brain tissue embolism associated with pre-slaughter stunning of cattle, International Conference on Veterinary Public Health and Food Safety, October 2004, Rome, Italy

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R.R. Coore, S. Love, J.L. McKinstry, H.R. Weaver, A. Philips, T. Hillman, C.R. Helps and M.H.Anil. (2004). Brain tissue embolism and captive bolt gun stunning of cattle. EU-RAIN Conference, Farm to Fork Food Safety: A call for common sense, Athens, Greece, May, 2004

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# THE SPINE

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# SHORT COMMUNICATIONS

## Neural embolism and cerebral venous drainage at stunning and slaughter

R. R. COORE, F. J. BARR, J. L. MCKINSTRY, M. H. ANIL

THE establishment of a link between bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt-Jakob disease in human beings (Will and others 1996) initiated a review of stunning and slaughter practices to prevent the contamination of carcases by tissues designated as specified risk material (SRM). In the UK, over 30 per cent of sheep are stunned before slaughter by a captive bolt gun (Anon 1997). Due to the continuing uncertainty over whether the UK sheep flock may harbour the BSE agent, equally stringent SRM controls are applied to sheep as to bovine carcases.

The continued use of captive bolt guns for stunning sheep and cattle in the UK has been questioned as investigations have demonstrated the risk of neural tissue embolism and carcase contamination after stunning (Anil and others 2001). Neural tissue emboli were detected in the jugular return of four of 15 sheep after the use of two types of captive bolt guns (Anil and others 2001). The presence of alternative cerebral drainage vessels in this species suggests that neural tissue emboli might also be detectable in other veins draining blood from the head.

The internal vertebral plexus (IVP) has been well described in both human beings (Groen and others 1997) and in a variety of animal species (Smuts 1977, Ghoshal and others 1981) including sheep (Rauhut 1962). The plexus originates at the foramen magnum from the cerebral venous sinuses and forms paired, ventrally situated interconnecting vessels, which extend posteriorly within and along the length of the vertebral canal. The IVP offers an alternative outflow for venous blood from the head that may, under certain conditions, exceed that of the jugular veins (Valdueza and others 2000, Zippel and others 2001).

During stunning and slaughter, an animal will undergo various postural changes starting with a normal standing position and changing to one of recumbency after stunning. Finally, the animal may be hoisted into a head-down position for slaughter by exsanguination or, alternatively, may be exsanguinated while recumbent. This short communication describes the effect of posture upon the venous outflow from the head in the context of stunning and slaughter, and the potential for the carriage of neural emboli.

Radiographic studies were performed on four adult cull sheep. The animals were anaesthetised with an intramuscular induction mixture (12 mg/kg bodyweight ketamine and 0·4 mg/kg bodyweight xylazine), and then intubated and maintained on halothane, oxygen and nitrous oxide. The angularis oculi vein, which runs medial and ventral to the eyes, was raised by digital pressure and cannulated with a 22 G 1 inch catheter (Otiva, Johnson and Johnson). A similar catheter was also placed in the contralateral vein. With the animals in a recumbent position, 10 ml of contrast medium (Omnipaque; Nycomed) was injected simultaneously into both veins via the catheters. At the same time, a dorsoventral radiograph was taken of the head and neck. A second dorsoventral radiograph was taken within 30 seconds of the first.

While maintaining anaesthesia, each animal was hoisted to assume a head-down position to mimic the effect of hoisting following stunning. The injections of contrast medium and the radiographs of the head and neck were then repeated. All four animals were euthanased humanely without recovery from anaesthesia.

The radiographic images obtained show the veins by which the contrast medium drained from the cerebral sinuses (Fig 1). In the recumbent position, the IVP is clearly visible, indicating a significant carriage of blood from the head, while the jugular veins are only faintly apparent, suggesting a reduced blood flow in these vessels (Fig 1a). The radiographs taken following hoisting of the sheep into a head-down position show distension of the jugular veins, while the IVP is not visible (Fig 1b).

*Veterinary Record* (2004) **155**, 86-87

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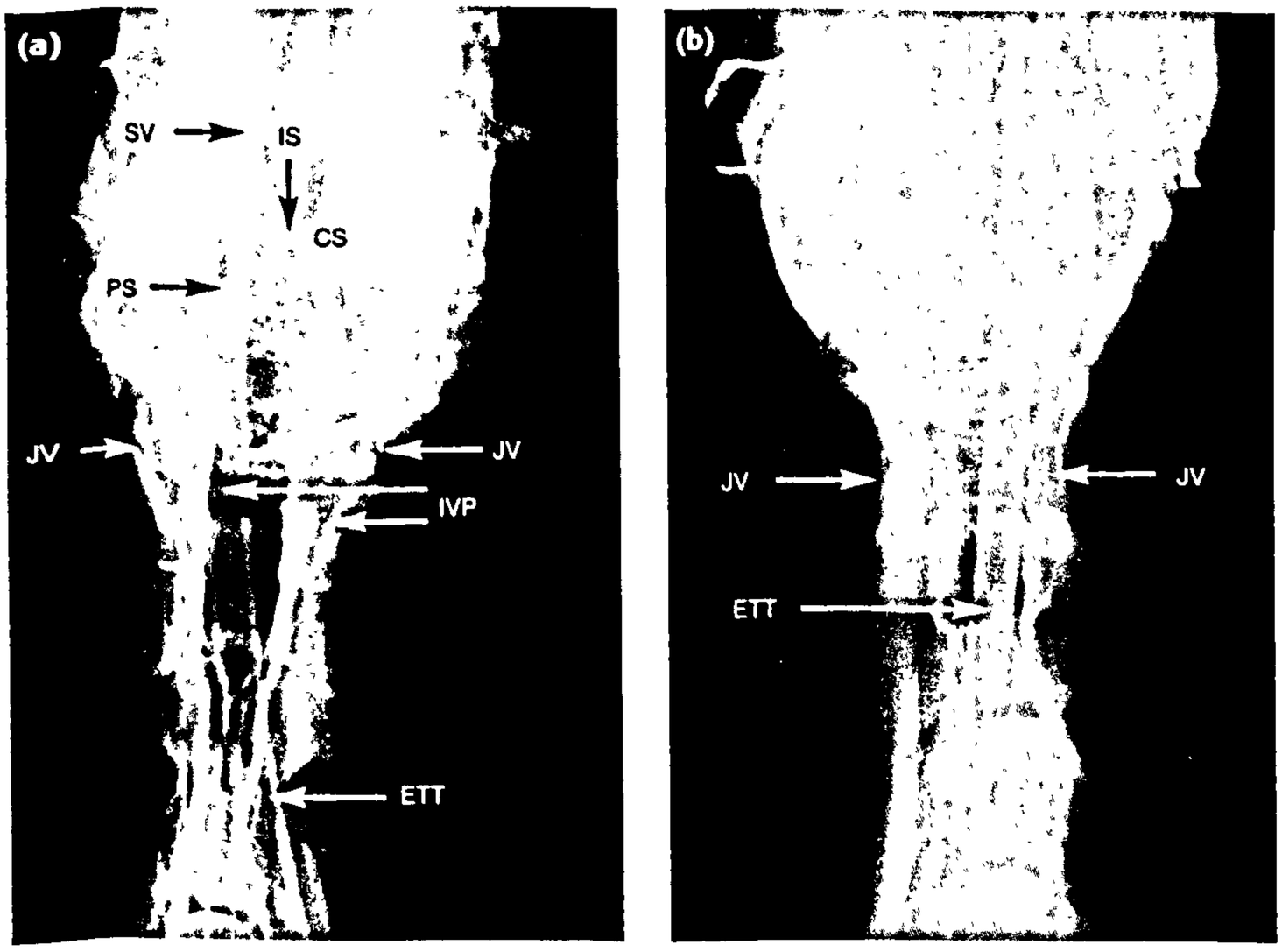


**FIG 1: Dorsoventral venogram of the head and neck of a sheep with the animal (a) in a recumbent position, and (b) hoisted into a head-down position, following the injection of 10 ml of contrast medium into both angularis oculi veins. SV Sphenopalatine vein, CS Cavernous sinus, PS Petrosal sinus, IVP Internal vertebral plexus, JV Jugular vein, ETT Endotracheal tube, SC Sinuum confluens, IS Intercavernous sinus**

The findings suggest that, following stunning using a captive bolt gun, the IVP may be capable of transporting neural emboli while the animal is in a recumbent position. This initial period following stunning lasts at least 15 seconds, which is within the time that neural emboli may be detectable in blood after captive bolt gun stunning (Anil and others 2001). After hoisting, it appears that the jugular veins will drain all the blood from the head, making it unlikely that, at this stage of the slaughter process, the IVP could contribute towards the carriage of neural emboli. As the jugular veins are themselves severed at exsanguination, any risk of carcase contamination by this route should be minimised.

Possible changes to cerebral venous drainage routes from the head as a direct consequence of captive bolt gun stunning may need to be examined in future studies. If further investigations indicate that the IVP may offer an additional route for the dissemination of neural tissue emboli after captive bolt gun stunning, a review of current stunning and slaughter practices may be required.

**ACKNOWLEDGEMENTS**

This work was funded by the UK's Food Standards Agency. The authors are grateful for the technical assistance of Mr Allen Jones.

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# ABSTRACTS

## Treatment of anal furunculosis with cyclosporin A and ketoconazole

NINETEEN dogs with anal furunculosis were treated with a combination of cyclosporin A and ketoconazole, and the signs resolved completely within three to 10 weeks. However, in seven of the dogs the signs recurred after remission periods ranging from one to six months. In some of the dogs the treatment had adverse effects, including hair loss, lethargy, vomiting and poor appetite, but none of them was considered serious. The results were as good as or better than the surgical alternatives, and there was an approximately 70 per cent saving in cost compared with using cyclosporin A alone.

O'NEILL, T., EDWARDS, G. A. & HOLLOWAY, S. (2004) Efficacy of combined cyclosporine A and ketoconazole treatment of anal furunculosis. *Journal of Small Animal Practice* **45,** 238-243

## Transient hydrops fetalis in pregnant dogs diagnosed by ultrasound

BETWEEN November 1999 and May 2002 a total of 161 bitches were diagnosed pregnant by ultrasound. Seventeen of them developed signs of hydrops fetalis during the pregnancy, but no pups were born with clinical signs of hydrops fetalis. One of the litters was aborted after the bitch developed systemic mastocytosis. Of the 95 pups in the other 16 litters seven were resorbed, eight were aborted and seven were stillborn. Eleven of the 73 pups born alive died within a few days and seven were born with congenital abnormalities. Pugs were significantly more likely to be affected than other breeds.

HOPPER, B. J., RICHARDSON, J. L. & LESTER, N. V. (2004) Spontaneous antenatal resolution of canine hydrops fetalis diagnosed by ultrasound. *Journal of Small Animal Practice* **45,** 2-8

*Journal of Food Protection, Vol. 67, No. 5, 2004, Pages 1050–1052*


Research Note

# Dissemination of Brain Emboli following Captive Bolt Stunning of Sheep: Capacity for Entry into the Systemic Arterial Circulation

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MS 03-386: Received 10 September 2003/Accepted 5 December 2003

## ABSTRACT

The epidemic of bovine spongiform encephalopathy in the United Kingdom and the recognition of a variant of Creutzfeldt-Jakob disease prompted revision of the guidelines for slaughter of cattle and sheep to prevent contamination of the edible parts of the carcass with central nervous system tissue. We previously showed that captive bolt gun stunning, which is routinely used for the slaughter of cattle and sheep, causes entry of fragments of central nervous system tissue into the jugular vein. To determine whether such tissue can traverse pulmonary capillaries to enter the systemic circulation, we introduced small volumes of brain tissue that had been disrupted by stunning with a captive bolt gun into the jugular vein of sheep sent for slaughter We examined aortic blood samples by immunocytochemistry for neurofilament and S100 proteins and by enzyme-linked immunosorbent assay for glial fibrillary acidic protein and found fragments of neurofilament- and S100-immunopositive central nervous system tissue in samples from 2 of 11 sheep and elevated glial fibrillary acidic protein in 6 sheep. Our findings suggest that central nervous system tissue that is dislodged during routine captive bolt gun stunning and slaughter of sheep can enter the systemic arterial circulation and that, in some cases, this method of slaughter of an animal infected with bovine spongiform encephalopathy would be likely to contaminate edible parts of the carcass with infective material.

Bovine spongiform encephalopathy (BSE) was first recognized in the United Kingdom in the mid-1980s *(14)*. There is now compelling evidence that the BSE agent is responsible for a variant of Creutzfeldt-Jakob disease first reported in 1996 *(15)*. The recognition of BSE and subsequently of its link to the variant Creutzfeldt-Jakob disease *(8)* led to the introduction of stringent measures to prevent the contamination of meat products by brain and spinal cord, the tissues with the highest potential for infectivity *(10)*. Although the controls reduce the risk of entry of BSE-infected material into the food chain, it remains unclear how effective they are in preventing contamination of edible parts of the carcass. This concern is relevant to sheep as well as cattle because it is still uncertain whether sheep in the United Kingdom might also be affected by the BSE agent *(12)*.

In the United Kingdom, most cattle and many sheep are stunned by captive bolt guns (CBGs). In 1996, U.S. researchers noted neural tissue in the pulmonary arteries of cattle stunned with an air-injection pneumatic gun *(6)*. Subsequently, Bauer et al. *(4)* identified neural emboli within the pulmonary arteries of 7 of 220 cattle carcasses. At the time, this finding was refuted in the United Kingdom *(13)*, but the occurrence of neural emboli after stunning with some types of CBG was subsequently confirmed *(3)*.

In previous studies of cattle and sheep subjected to conventional captive bolt stunning, we found neural emboli in jugular venous blood *(1–3)* but not in single samples of blood taken from the aorta in a small number of animals *(1)*. We have now further explored the possibility that entry into the jugular venous return of central nervous system (CNS) tissue carries a risk of dissemination within the systemic arterial circulation, risking the contamination of edible parts of the carcass.

## MATERIALS AND METHODS

**Animals.** Twelve adult sheep were used: 11 experimental animals and 1 control. The 11 experimental animals were anesthetized with a mixture of ketamine (12 mg/kg, Fort Dodge, Southampton, UK), and xylazine (0.4 mg/kg, Bayer, Bury St. Edmunds, UK) given intravenously. Once the animals were deeply anesthetized, the jugular vein was exposed and a catheter introduced. Anesthesia was maintained by intravenous infusion of a 100:3:10 mixture of 10% guaiphenesin (Chassot [UK] Ltd., Preswick, UK), 2% xylazine, and 10% ketamine. An arterial catheter was advanced through one of the carotid arteries into the proximal aorta. A previously prepared 10-ml suspension of brain tissue fragments in normal saline (see below) was then injected through the jugular catheter At the same time that the suspension was injected, the animal was stunned with a pneumatically activated

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penetrating CBG (Cash Ramrod, Accles and Shelvoke, Birmingham, UK), and blood collection was initiated through the aortic catheter. The collection of blood was continued until all flow had ceased 3 to 11 min later. The volume collected over each successive 1-min period was divided into aliquots with 20 ml of citrate anticoagulant in 250-ml bottles. Postmortem examination confirmed correct placement of the aortic catheter in every case.

**Preparation of brain tissue suspensions.** Loose brain tissue created by previous stunning of an animal with a penetrating CBG was collected through the bolthole of a fresh carcass by flushing with normal saline followed by aspiration with a 10-ml syringe. The tissue was suspended by vigorous agitation in normal saline at a ratio of 1 g tissue to 9 ml saline. A 10-ml sample of suspension therefore contained approximately 1 g of brain tissue. Our previous studies suggest that this is a conservative estimate of the amount of brain tissue that can be dislodged from the cranial cavity by a CBG *(1)*. Our aim in obtaining and suspending the brain tissue in the manner described was to achieve a volume of CNS tissue and range of particle sizes comparable to the embolic showers produced by a CBG.

**Positive controls.** One sheep was stunned by CBG and five 100-ml aliquots of blood were collected without prior injection of brain tissue into the jugular vein. A suspension of brain tissue was prepared as described above. Dilutions of brain volumes of 0.1, 0.5, 1.0, 5, and 10 ml of suspension were added to the blood aliquots.

**Negative controls.** Negative controls comprised jugular venous blood samples taken from sheep before stunning and injection of brain suspension.

**Preparation of aortic blood samples for assays.** Buffy coat fractions were prepared as described *(3, 9)*. For enzyme-linked immunosorbent assays (ELISAs), 3 ml of each aliquot was frozen rapidly in liquid nitrogen and stored at −70°C until just before assay. The remaining volume was fixed in an equal volume of 20% formalin for immunocytochemistry.

**ELISA.** A capture ELISA for glial fibrillary acidic protein (GFAP), a specific marker of CNS tissue *(11)*, was used. The method followed that of an earlier study *(7)*, except that 75 μl of buffy coat from each aliquot was mixed with 75 μl of phosphate-buffered saline and 0.5% Triton X-100 for the assays. Duplicate 50-μl samples of this mixture were assayed.

**Immunocytochemistry.** The immunocytochemical methods were as previously described *(9)*. Samples of formalin-fixed buffy coat were pelleted and embedded in paraffin wax Cytoblocks (Shandon, Runcorne, UK). Sections (5 μm) were cut in triplicate at three levels through the blocks. The adjacent sections were stained with hematoxylin and eosin or immunostained by a standard strepatavidin-biotin-peroxidase method for neurofilament protein (Dako M0762, Dako, Ely, UK) or S100 protein (Dako Z0311). In addition to the controls described above, each immunocytochemical run included positive samples from previous studies *(9)* and sections immunostained with omission of primary antibody.

## RESULTS AND DISCUSSION

**GFAP ELISA.** The results of the ELISA showed that GFAP was absent from all blood samples taken before injection of brain tissue and was detected in all of the positive control samples. Levels were elevated in aortic blood from 6 of 11 animals tested (Table 1). In five animals, GFAP was detectable within 60 s of injection of brain suspension and in all subsequent samples until blood flow ceased. In the sixth animal, GFAP was detected in only the third, final sample.

TABLE 1. *Analysis of samples by glial fibrillary acidic protein (GFAP) ELISA*

| Time (min)[a] | Results by sheep[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | h | i | j | k | l |
| 1 | + | + | ND | + | ND | + | + | ND | ND | ND | ND |
| 2 | + | + | ND | + | ND | + | + | ND | ND | ND | ND |
| 3 | + | + | ND | + | ND | + | + | ND | ND | + | ND |
| 4 | | | ND | + | ND | + | + | ND | ND | | ND |
| 5 | | | ND | + | ND | + | + | ND | ND | | ND |
| 6 | | | ND | + | ND | + | | ND | ND | | ND |
| 7 | | | ND | + | ND | | | ND | ND | | ND |
| 8 | | | ND | + | ND | | | ND | ND | | ND |
| 9 | | | + | | | | | ND | ND | | ND |
| 10 | | | + | | | | | | | | ND |
| 11 | | | + | | | | | | | | |

[a] Time following injection of brain homogenate and stunning by CBG.

[b] Each animal was designated a letter from a through l. The absent sheep (g) was the positive control sample. +, positive for GFAP; ND, not detectable.

**Microscopy and immunocytochemistry.** Immunocytochemical analysis demonstrated that CNS tissue was absent from all blood samples taken before injection of brain tissue and was detectable by immunocytochemistry for both S100 protein and neurofilament protein in all of the positive control samples. Multiple buffy coat samples from two animals—S(h), S(k)—yielded Cytoblock sections that contained tissue immunopositive for S100 and neurofilament proteins (Fig. 1).

The lungs are effective filters of cellular embolic material, such as metastatic tumors *(5)*. Although CNS emboli are known to enter the jugular veins *(2, 3)* and pulmonary arteries *(6)* after stunning with a CBG, it has not previously been shown that such emboli can pass through the pulmonary vasculature to enter the aorta. This is clearly a critical determinant of the risk that stunning by CBG might disseminate $PrP^{Sc}$ within the edible tissues of a BSE-infected animal. In previous studies, we suggested that some of the fragments of CNS tissue in the jugular venous blood of cattle stunned with a CBG were small enough to traverse the pulmonary capillary bed *(2, 3, 9)*. The present findings indicate that this might indeed occur and, in addition, demonstrated that emboli as large as 20 μm in diameter can pass through the ovine pulmonary capillary bed to enter the arterial circulation.

The reliability and specificity of the methods used in this study have been evaluated and validated extensively *(2, 3, 9)*. The discrepancies between the results of immunocytochemistry and ELISA in some animals probably relate to the limited sampling of buffy coat in these assays. In total, less than 1% of the buffy coat in any aliquot was sampled for immunocytochemistry or ELISA, and although

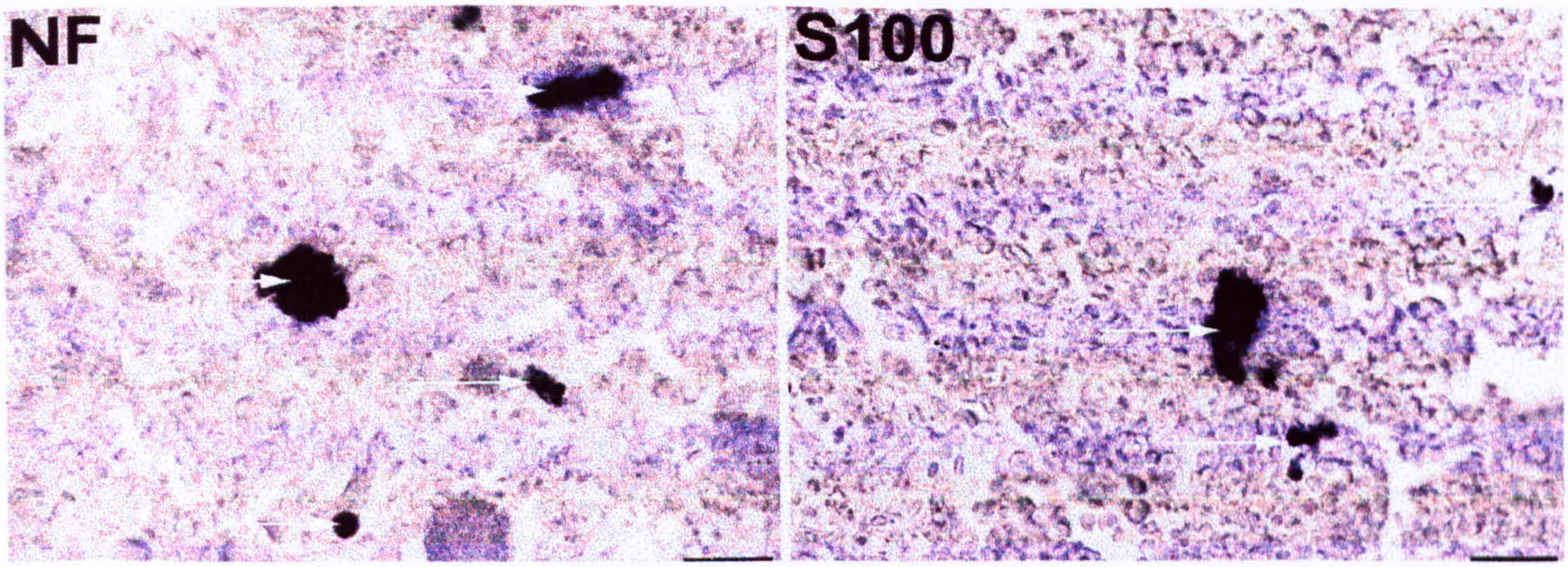


FIGURE 1. *Cytoblock sections of buffy coat from a sheep with CNS tissue in aortic blood samples. The sections have been labeled for neurofilament protein (NF) and S100 protein. Both sections contain fragments of immunopositive CNS tissue. Bars = 20 μm.*

immunocytochemistry was performed on sections cut at several levels, the intervening material was not examined. It is therefore inevitable that the immunocytochemical findings will have tended to underestimate the number of positive samples. Furthermore, because the neural emboli are particulate in nature, the blood samples cannot be considered to be homogeneous solutions of GFAP, and ELISA might also underestimate the number of positive samples.

ELISAs on sequential aliquots of blood in the six animals with positive assays indicated that neural tissue continued to be released from the pulmonary vasculature into the aorta for as long as the heart continued to beat, although the initial bolus had been administered over a period of only a few seconds. The delayed passage of some of the embolic tissue through the lungs probably reflected partial obstruction by the pulmonary capillaries. In contrast, some embolic tissue passed through the lungs into the systemic circulation within a minute of its introduction into the jugular vein. Neural emboli can enter jugular veins within 30 s of stunning with a CBG *(3)*, and in the context of stunning and slaughter of livestock in abattoirs, our results suggest that there would be sufficient time for emboli to pass through the lungs and into the systemic circulation before exsanguination and cardiac arrest.

The present study has confirmed the potential for systemic embolization of CNS tissue. Further work is necessary to improve the sensitivity of the assays, to determine the incidence of neural embolism in larger cohorts, and to assess the risk in cattle as well as sheep. Although BSE is declining in the United Kingdom, at least in cattle, it is increasing in incidence in other countries, and it is important to continue to try to minimize the risk that stunning and slaughter of subclinically infected animals will cause prions to enter the human food chain. To this end, it is necessary to explore alternative methods of stunning, existing or novel, that do not carry the risk of neural embolism.

## ACKNOWLEDGMENT

This work was supported by the UK Food Standards Agency.

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FOODBORNE PATHOGENS AND DISEASE
Volume 1, Number 4, 2004


## Short Communication

# Frequency of Brain Tissue Embolism Associated with Captive Bolt Gun Stunning of Sheep

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## ABSTRACT

**In accordance with controls instituted to protect the consumer from meat potentially infected with bovine spongiform encephalopathy (BSE), brain tissue emboli caused by the use of captive bolt gun (CBG) stunning have been identified as a potential public health risk that requires further investigation. As the natural occurrence of BSE in sheep remains uncertain we have investigated the frequency of brain tissue embolism associated with stunning by two types of CBG that are in commercial use in the United Kingdom. Blood samples collected from sheep following stunning were analysed by ELISA as well as by a combination of microscopy and immunocytochemistry. The combined positive results from each method of sample analysis were used to determine the frequency of brain tissue embolism in the sample population. The frequency of brain tissue embolism was found to be 23% in sheep stunned with a cartridge-activated CBG (95% confidence interval of 15.8–32.2%) and 14% in those stunned with a pneumatically activated CBG (95% confidence interval of 8.5–22%). The frequency of brain tissue embolism associated with the use of CBG stunning may represent a significant source of carcass contamination with brain tissue.**

## INTRODUCTION

ALTHOUGH EXTENSIVE INVESTIGATIONS have not revealed naturally acquired bovine spongiform encephalopathy (BSE) in sheep (Baylis et al., 2002), the UK government has implemented controls to reduce any potential risk to the human population should BSE be detected in the sheep population in the future.

Brain tissue has previously been detected in jugular blood samples from two of 15 sheep (13.3%) that had been stunned with a conventional penetrating captive bolt gun (CBG) and in a further two of 15 animals that had been stunned with a pneumatically-activated penetrating CBG (Anil et al., 2001, 2002). CBGs are used to stun around 38% of sheep in the UK and brain tissue embolism may present a significant risk factor for carcass contamination with tissues of potential high infectivity (Anil et al., 2001). In this paper, we present the results of work undertaken to examine the frequency of brain tissue emboli present in jugular venous blood obtained from 200 sheep stunned using either a cartridge or pneumatically activated penetrating CBG.

### *Sample collection*

Two hundred adult sheep of varying age were sampled for brain tissue embolism following stunning by one of two types of CBG. All sheep were stunned by an experienced slaughter-man according to standard commer-

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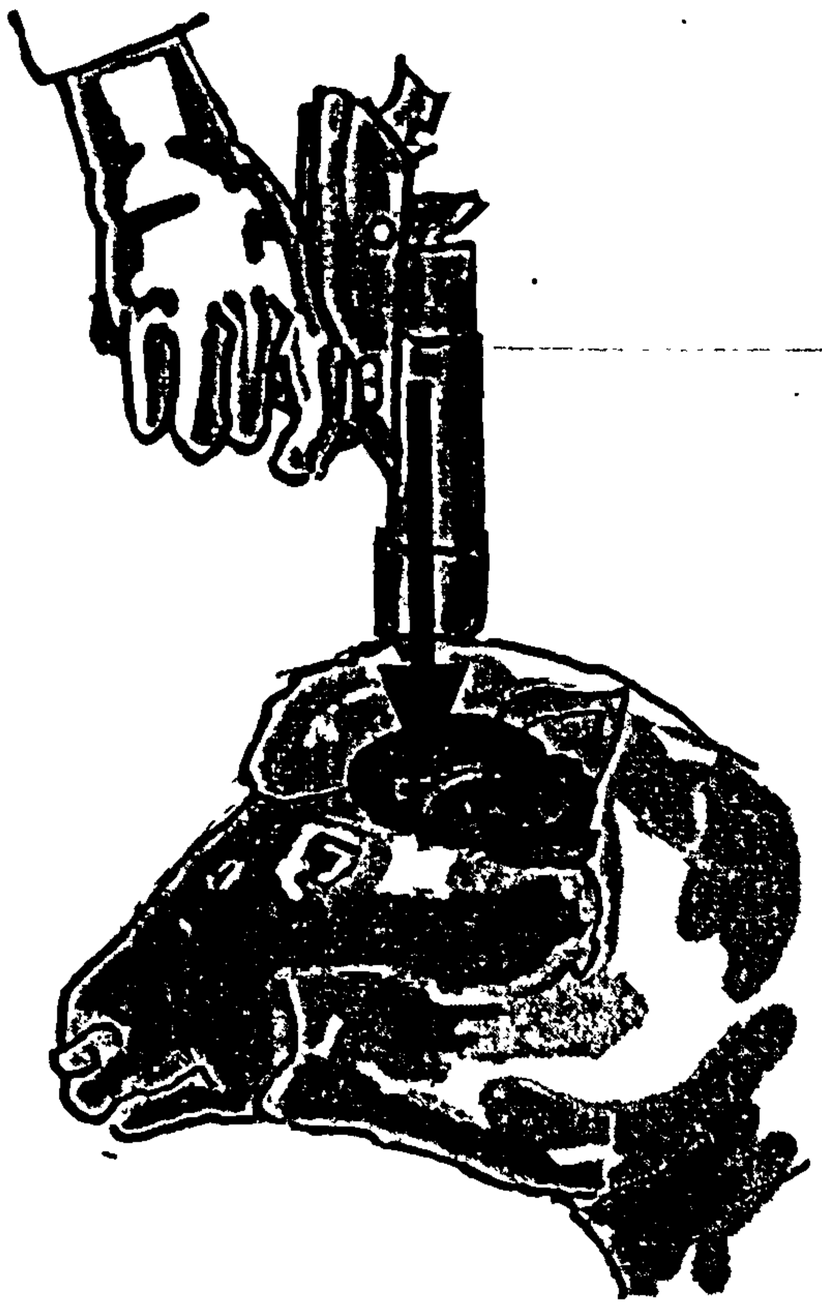

FIG. 1. The anatomical landmarks used for identifying the correct stunning position followed that for polled sheep. The muzzle of the CBG was placed on the highest point of the head on the midline aiming straight down before activation of the CBG.

cial practice in the UK (Fig. 1). The animals were first anaesthetized by intra-muscular administration of a ketamine (12 mg/kg; Fort Dodge, Southampton, UK) and xylazine (0.4 mg/kg; Bayer, Bury St. Edmunds, UK) mixture. Anaesthetic depth was maintained by intravenous infusion of a 100:3:6 mixture of 10% guaiphenesin (Chassot Ltd, Preswick, UK), 2% xylazine, and 10% ketamine. After sufficient anaesthetic depth was achieved, a plastic cannula was advanced in a cranial direction into each jugular vein and firmly secured in place by ligatures. A 10-mL pre-stun sample of blood was taken from the jugular cannulas into a collection tube containing citrate anti-coagulant (3.8% citrate, pH 5.2).

Half of the sheep were stunned with a cartridge-activated penetrating CBG (Temple Cox Mark X, Accles & Shelvoke, Birmingham, UK), and half were stunned with a pneumatically-activated penetrating CBG without air-injection facility (Cash Ramrod, Accles & Shelvoke, Birmingham, UK). Simultaneously with stunning, blood collection was initiated from the pre-placed jugular cannulas and continued over one minute. Samples were taken at 10-sec intervals into six separate plastic 250-mL bottles containing citrate anticoagulant (3.8% citrate, pH 5.2). Following sample collection, the animals were slaughtered by exsanguination according to normal commercial practice.

*Processing of blood samples*

The samples were placed in a pre-cooled centrifuge (Hi-spin 21, MSE, UK; 5°C) and centrifuged at 800 × *g* for 30 min. The buffy coat from all samples was isolated by suction of the greater quantity of red bloods cells and plasma, and then three 1-mL aliquots of the isolated buffy coat fraction were frozen at −30°C and retained for assay by ELISA. Samples of buffy coat were also retained for analysis by microscopy and immunocytochemistry.

*Positive controls*

For positive controls, a series of five two-fold dilutions of macerated brain tissue in blood was prepared and sent for analysis by ELISA and by microscopy and immunocytochemistry.

TABLE 1. ANIMALS POSITIVE FOR BRAIN TISSUE EMBOLISM BY ANALYSIS OF BLOOD SAMPLES

| | *No. of positive animals stunned by cartridge-activated CBG (n = 100)* | *No. of positive animals stunned by pneumatically-activated CBG (n = 100)* |
|---|---|---|
| GFAP ELISA | 14 | 10 |
| Microscopy and immunocytochemistry | 18 | 13 |
| Total positive animals | 23[a] | 14[a] |

[a]Nine animals were confirmed positive for brain tissue embolism by both assays.

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*itive controls*

egative controls included jugular blood sam-aken prior to stunning from all animals.

*ure ELISA for GFAP*

ial fibrillary acidic protein (GFAP) has pre-sly been used as a sensitive marker of cen-nervous system tissue (Helps et al., 2002). previously described ELISA for GFAP ps et al., 2002) with minor modifications used to analyse all samples. Modifications assay included the initial addition of 75 buffy coat from each aliquot to 75 $\mu$L of 0.5% Triton-X 100 to break down any em-material and achieve a homogeneous so-n. Duplicate 50-$\mu$L samples of this mixture then assayed according to the described ocol.

*oscopy and immunocytochemistry*

The method for sample processing and analy-has been previously described (Love et al., 00). Briefly, samples of buffy coat were fixed ormalin, pelleted, and embedded in paraffin Cytoblocks. 5-$\mu$m-thick sections were then at multiple levels through the block. Adja-sections were stained with haematoxylin eosin or immunostained by a standard ptavidin-biotin-peroxidase method for neu-lament protein (Dako, Ely, UK) or S100 pro-n (Dako, Ely, UK). Samples were regarded as sitive if the Cytoblock sections included frag-nts of tissue that were immunopositive for neurofilament protein and S100 protein.

## RESULTS AND DISCUSSION

he frequency of brain tissue embolism in p stunned by the cartridge-activated, pene-ing CBG was 23% (95% confidence interval 5.8–32.2%). The frequency of brain tissue bolism following the use of the pneumati-y activated CBG was 14% (95% confidence rval of 8.5%–22%). These results represent combined positive results obtained by AP ELISA and analysis by microscopy and munocytochemistry (Table 1). No statistical ference was found between the frequencies obtained from each CBG group ($p = 0.174$, Chi-squared analysis).

The detection limit of the ELISA and the microscopy and immunocytochemical analysis was found to be 156 $\mu$g/mL (wet weight brain per ml blood) and 312 $\mu$g/mL (wet weight brain per ml blood), respectively. All negative control samples were correctly identified by both methods of sample analysis. The intra-assay and inter-assay coefficient of variation of the GFAP ELISA was 13% and 17%, respectively.

Comparison of the positive results obtained by ELISA and by microscopy and immunocytochemistry demonstrates a greater disparity than previously found in similar studies (Anil et al., 2001, 2002). This discrepancy may relate to the small volumes of venous blood analyzed by each test. For practical reasons, <1% of each aliquot obtained over a 10-sec period could be analyzed, and it is therefore likely that both methods underestimated the number of weakly positive aliquots. However, by combining the two methods, we have tried to minimize the number of false negative results. The frequency of brain tissue embolism found in this study suggests that a significant proportion of sheep carcasses entering the food chain after CBG stunning risk contamination with brain tissues. If BSE were to be identified in farmed sheep, the use of CBG stunning in this species might pose a risk to the consumer.

## ACKNOWLEDGMENTS

This work was supported by the UK Food Standards Agency (MO 3013). Approval was obtained for the study from the UK Home Office and Intervention Board. The technical assistance of Justin McKinstry, Helen Weaver, Andy Phillips, Tim Hillman, and Marion Hiles is acknowledged.

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*Journal of Food Protection, Vol. 68, No. 4, 2005, Pages 882–884*


Research Note

# Brain Tissue Fragments in Jugular Vein Blood of Cattle Stunned by Use of Penetrating or Nonpenetrating Captive Bolt Guns

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MS 04-217: Received 11 May 2004/Accepted 14 August 2004

## ABSTRACT

Although the incidence of bovine spongiform encephalopathy in cattle continues to decline in the United Kingdom, it remains important to maintain vigilance of all potential routes of transmission of infection to humans. Initial studies have demonstrated a potential risk of carcass contamination with brain tissue following the use of captive bolt gun stunning in cattle. The objective of this study was to further explore these initial findings particularly in regard to captive bolt guns currently in use in the United Kingdom. Brain tissue fragments or elevated levels of a marker protein for brain tissue were detected in venous blood samples from 4% (95% confidence interval, 1.6 to 9.8%) of cattle stunned by penetrating captive bolt gun and from 2% (95% confidence interval, 0.6 to 7%) of those stunned by nonpenetrating captive bolt gun.

The controls instituted to eliminate bovine spongiform encephalopathy from United Kingdom cattle have been successful in causing a steady decline in reported cases of the disease *(5)*, and a range of further measures has been taken to reduce the possibility of transmission from the remaining bovine spongiform encephalopathy cases to humans *(3)*. Nevertheless, it remains important to maintain vigilance and to explore and properly assess all potential routes by which consumers might be exposed to the bovine spongiform encephalopathy agent.

To maintain welfare standards, most cattle in the United Kingdom are stunned with a captive bolt gun (CBG) before slaughter *(4)*. The use of CBGs for stunning cattle has previously been shown to introduce fragments of brain tissue into the venous circulation in some cases, raising questions as to the risk of contamination of the carcass with potentially bovine spongiform encephalopathy–infected tissues *(1, 2)*. Investigations demonstrated brain tissue fragments in the jugular blood of 4 of 15 cattle sampled following the use of a pneumatically operated air-injection penetrating CBG and in a single cow stunned with a conventional cartridge-activated penetrative CBG followed by pithing *(1, 2)*. As a consequence of these and other findings, the practice of pithing is now banned in the United Kingdom and European Union, and air-injection type CBGs are no longer used. Based on analysis of this earlier work *(1, 2)*, the United Kingdom Food Standards Agency recommended further investigation of the potential of stunning methods to introduce brain tissue into the venous circulation.

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## MATERIALS AND METHODS

**Sample collection.** Two hundred cattle were sampled at a designated abattoir used for culling cattle over 30 months of age, with the permission of the United Kingdom Home Office and Intervention Board, in batches of 6 to 10 animals over a 1-year period. Anesthesia was induced in each animal by administering an intravenous bolus of xylazine (0.4 mg/kg; Bayer, Bury St. Edmunds, UK) and ketamine (12 mg/kg; Fort Dodge, Southampton, UK). Anesthetic depth was maintained with further doses of a 100:3:6 anesthetic mixture of 10% guaiphenesin (Chassot [UK] Ltd, Preswick, UK), 2% xylazine, and 10% ketamine. Under deep anesthesia an incision was made over and into each jugular vein, and 14-gauge Foley catheters were advanced in a cranial direction into each jugular vein lumen and secured in place with ligatures. The catheter balloon cuffs were then inflated to ensure collection of all blood returning in the jugular veins. A 10-ml prestun sample was collected at this point into a test tube containing citrate anticoagulant (3.8% citrate, pH 5.2).

One hundred animals were stunned with a penetrating cartridge-operated CBG (Cash 8000 Cowpuncher, Accles & Shelvoke, Birmingham, UK) using a three-grain cartridge. A nonpenetrating cartridge-operated CBG (Cash 9000 Magnum Knocker, Accles & Shelvoke) with a six-grain cartridge was used on the second group of 100 animals. Immediately after stunning, blood collection was initiated from the preplaced jugular catheters. Blood sampling continued at 10-s intervals over 1 min into six 250-ml collection bottles containing citrate anticoagulant (3.8% citrate, pH 5.2). This collection period was chosen to include the average time that may elapse between stunning and exsanguination. Following sample collection the animals were shackled and exsanguinated according to normal commercial practice.

**Positive controls.** A quantity of macerated fresh bovine brain tissue was used to prepare a twofold serial dilution in blood over concentrations ranging from 10 mg/ml to 0.6 μg/ml. The dilution

eries was repeated on five separate occasions, and the resulting ontrol samples were processed and analyzed by methods identical o those used on the study samples.

**Negative controls.** A dilution series without added brain tisue was sent for processing and analysis along with all samples ıken prior to stunning each animal.

**Processing of samples.** The samples were placed in a prepoled centrifuge (Hi-spin 21, MSE, Crawley, Sussex, UK) at 5°C ıd spun at 800 × *g* for 30 min. The buffy coat was removed ıd 3 ml retained for assay by glial fibrillary acidic protein enyme-linked immunosorbent assay (GFAP ELISA); the remaining olume was fixed in an equal volume of 20% formalin to be ıalyzed by microscopy and immunocytochemistry.

**Capture ELISA for GFAP.** GFAP is the principle 8- to 9-m intermediate length filament found in astrocytes of the central ervous system and has previously been used as a sensitive marker of central nervous system tissue *(7, 10)*. A previously described LISA for GFAP *(7)* with minor modifications was used to analyze all samples. Modifications to the assay included the initial ddition of 75 μl of buffy coat from each aliquot to 75 μl of hosphate-buffered saline, 0.5% Triton X-100 to break down any ragments of brain tissue and achieve a homogeneous solution. uplicate 50-μl samples of this mixture were then assayed according to the described protocol *(7)*.

Plates were incubated overnight at 4°C and read at wavelengths of 405 and 492 nm. A curve was generated from a serially liluted standard that allowed quantification of the results.

**Microscopy and immunocytochemistry.** A previously described method *(8)* was used to analyze samples taken between 20 and 40 s after stunning from each animal, since previous studies indicated that during this interval most of the dislodged brain tissue fragments enter the jugular venous return *(1, 2)*. Buffy coat samples were fixed in formalin, pelleted by centrifugation, and finally embedded in paraffin wax cytoblocks. Sections 5-μm thick were then cut at multiple levels through the block. Adjacent sections were stained with hematoxylin and eosin or were immunostained by a standard streptavidin-biotin-peroxidase method for neurofilament proteins (Dako M0762, Ely, Cambridgeshire, UK) and S100 protein (Dako ZO311, Ely). A positive result was defined by the presence of tissue fragments in the Cytoblock sections that reacted with both antibodies.

## RESULTS AND DISCUSSION

Analysis by microscopy and immunocytochemistry demonstrated small fragments of brain tissue in blood samples taken from three cattle stunned by penetrating CBG and in blood samples from a further two cattle stunned by nonpenetrating CBG. In addition, blood samples from two cattle stunned by penetrating CBG showed elevated levels of GFAP. The combined results from ELISA and from microscopy and immunocytochemical analysis indicate that 4% (95% CI, 1.6 to 9.8%) of cattle stunned by penetrating CBG and 2% (95% CI, 0.6 to 7%) stunned by nonpenetrating CBG were found to have brain tissue fragments and or elevated levels of GFAP in jugular blood samples. The agreement between the results of the complementary detection methods used in this study was not as consistent as that seen in previous work *(1, 2)*. Explanations for this discrepancy may relate to the small sample volumes analyzed by each method in combination with the nonhomogeneous

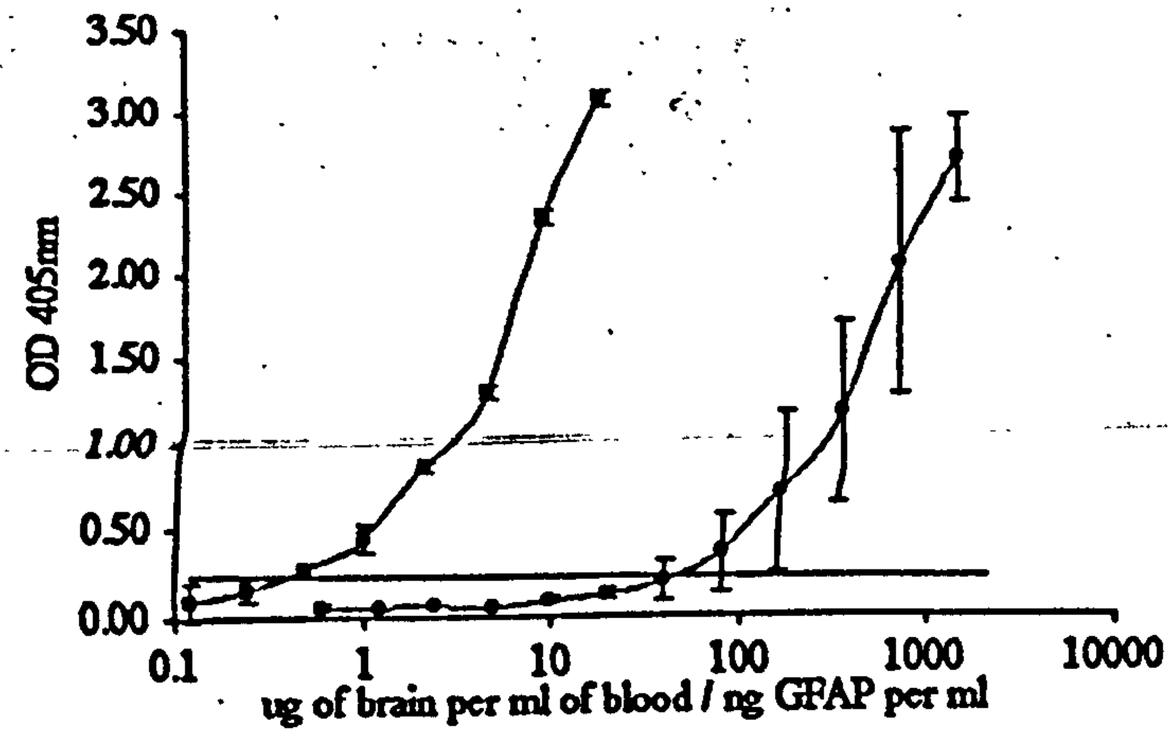


FIGURE 1. *Standard curve for the GFAP ELISA (■) and a parallel curve of dilutions of brain tissue in blood (●). Each point represents the mean ± standard deviation (SD) of five replicate samples that were assayed in duplicate. The positive and negative threshold (−) was calculated by the addition of the mean of 30 prestun optical density (OD) values and 5 SD.*

nature of the samples in which brain tissue fragments were present. An aliquot of blood without particulate brain tissue may be taken from an ELISA-positive sample, and conversely the presence of an occasional fragment of brain tissue in the buffy coat may not always be associated with elevated levels of GFAP in the liquid fraction of the sample.

The ELISA of the twofold serial dilutions of brain tissue in blood undertaken as part of the assay validation generated a curve that was parallel to that of the GFAP standard (American Research Products, Belmont, Mass.) (Fig. 1). The minimum detection limit of brain tissue in blood was found to be 156 μg/ml (wet weight brain per milliliter of blood) by ELISA and 312 μg/ml (wet weight brain per milliliter blood) by the complementary method of microscopy and immunocytochemistry.

The presence of brain tissue in blood samples taken after the use of the penetrating CBG without subsequent pithing has not previously been demonstrated and is particularly significant as this type of CBG is presently used by the majority of abattoirs in the United Kingdom and European Union. The finding of a 4% frequency in the sample population may equate to as many as 80,000 animals when extrapolated to the total number of cattle slaughtered annually in the United Kingdom *(6)*. The demonstration of brain tissue fragments in venous blood associated with the use of nonpenetrating CBG stunning casts doubt on the advisability of using these CBGs as a safer alternative to the use of penetrating CBGs. Recently, the European Commission initiated a review and consultation process with stakeholders on stunning methods for cattle as part of a recent project called SAFESTUN (accompanying measures QLK2-CT-2002-30531). A technical seminar held in 2003 considered alternative methods to CBG stunning that included modifications to the current designs of CBGs, as well as the use of novel methods such as transcranial magnetic stimulation. A final report of this project was submitted to the European Commission in 2004 *(9)*. Further studies of current stunning methods for cattle are needed to assess the quantity and potential for dissemination of brain

tissue fragments and proteins in the peripheral circulation, organs, and muscles of the carcass.

## ACKNOWLEDGMENT

This work was supported by the UK Food Standards Agency (Project no. MO 3012).

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