

Spongiform Encephalopathy Advisory Committee

Information paper

Date: 30 Aug. 2002

Title of Paper Update on project M03016: Determination of abnormal prion protein in the milk of cattle experimentally infected with the BSE agent.

Issue At the June 2002 meeting of SEAC the Committee requested an update on project M03016. This is provided in the attached paper.

NB: It should be noted that analysis of milk samples from cows experimentally infected with BSE has not yet commenced.

Action Required from Committee

1. To note the progress of the study with regard to the VLA Quality Systems, Milk sample collection and validation of the methods of analysis.
2. To note the issues of concern to the FSA and FSA action
3. To invite SEAC to provide written comment on/or endorsement of the FSA program for future work on:
 - a) this phase of the work and,
 - b) the whole project.
3. To comment on the significance that can be placed on the results from this phase of the study (i.e. negative or positive or inconclusive results).
4. To advise whether this study should be considered further by a sub-group of SEAC.

List of material attached

1. [An information paper](#) providing an update of progress on the validation of the methods of analysis for the determination of the abnormal prion protein in the cellular fraction of milk from cattle experimentally infected with the BSE agent (project M03016).
2. ANNEX I: The latest revision of the proposal for the above project, which includes the additional validation of the methods of analysis and milk sampling (3rd lactation)
3. ANNEX II: The protocol for the collection of milk samples to be used in the milk bioassay study the Food Standards Agency is planning.

Project M03016: DETERMINATION OF ABNORMAL PRION PROTEIN IN THE MILK OF CATTLE EXPERIMENTALLY INFECTED WITH THE BSE AGENT.

1. Introduction.

At the June 2002 meeting of SEAC, the committee requested a progress report on the research that the Food Standards Agency has commissioned at the Veterinary Laboratory Agency on Project **M03016: Determination of abnormal prion protein in the milk of cattle experimentally infected with the BSE agent** .

SEAC should note that this report covers only the development and validation of the analytical methods that will be used to establish if the BSE prion can be detected in the cellular fraction of milk collected from dairy cows experimentally infected with the BSE agent.

The Agency is fully aware of the need to ensure that the data obtained from the study is robust and is able to withstand critical review. With that in mind the Agency has discussed the progress of the study with the contractor, emphasising the importance of the work, the validity of the results and the need to ensure that there is a clear audit trail covering the sampling, storage, transport, analysis and reporting of the results.

2. Progress

Quality Systems. The VLA is working towards the implementation of a unified QA system across the VLA site in compliance with the VLA Quality Framework Document. Each department is being audited for compliance as they complete the implementation of each phase. The Department of TSE Molecular Biology, which is undertaking the study, has recently completed the implementation of the Quality Framework (sample handling) phase. The internal (VLA) audit of this phase will be completed during the early Autumn.

Sample collection. Milk samples are being collected from dairy cows experimentally infected with the BSE agent (DEFRA research project SE1736), and this is being conducted at ADAS Drayton. ADAS personnel are

responsible for the milk sampling, labelling, recording and storage and this is being carried out in accordance with protocols provided by the VLA. This phase of the study together with the subsequent handling, transport and storage of the samples (at ADAS Drayton and the VLA) is being audited by the VLA QA unit.

Methods of analysis. SEAC should note that in the true definition of “method validation”, the methods used for the analysis of the milk samples can not be fully validated. This is because neither a reference sample of PrPsc or a “incurred PrPsc” milk sample are available. The assay “*validation*” therefore has to rely on the use of milk samples that have been spiked with BSE +ve cattle brain or BSE –ve cattle brain (control).

The method “*validation*” has focused on the Prionics test, the Bio-Rad (CEA) test and a Hybrid - Prionics/Bio-Rad test. Ongoing work is determining the sensitivity of the methods i.e. limit of detection and limit of determination in terms of BSE +ve cattle brain equivalents and establishing the reproducibility and repeatability of the methods.

When completed the VLA will provide the Agency with documents describing in detail the method “*validation*” together with the “*validation*” data. The VLA will also provide the Agency with a Standard Operating Procedure (SOP) for the analysis of the milk samples. The analysts undertaking the analysis of the experimental milk samples will follow this SOP. In addition they will not know the identity/origin of the samples being analysed and these will also include both BSE +ve and BSE –ve (control) samples.

3. Issues of concern to the FSA

Quality systems and data quality: Given the importance of the results of this study, it is imperative that there is a clear and well-documented audit trail. The VLA, Department of TSE Molecular Biology will most likely achieve full compliance with regard to sampling and document handling, during the next few months. Despite this the Agency considers there is a need to provide an independent verification of compliance with the VLA Quality Framework Document and to audit of the milk collection, handling and analysis trail.

“Validation” of the methods of analysis: Although a considerable amount of “validation” data has already been generated, further data is required to complete this phase of the study. Additional information supporting the validation of the analytical methods will be generated during the course of the analysis of the samples. However despite this the Agency consider that the methods of analysis should be “validated” as fully as possible before proceeding with the analysis of the experimental milk samples.

SEAC members will need to be aware that although the current method “validation” exercise is reliant on the spiking of milk samples with BSE affected brain material, it is quite likely that the PrPsc present in affected brain could differ from that which may be present in milk. In affected BSE brain the PrPsc is largely present as an insoluble aggregate, whilst in body fluids (e.g. milk) the PrPsc is likely to be more disperse, non-aggregated and quite possibly in a soluble form. The effect of this on the assays will need to be established, particularly with regard to the relative susceptibility of the two forms of PrPsc (soluble and insoluble/aggregated) and normal PrP to protease treatment.

4. FSA Action

The relationship between abnormal prion levels and BSE infectivity are unclear and further evaluation of the results of this work and their implications is envisaged. Given the above concerns and bearing in mind the potential implication of the results of this study the Agency is pursuing the following action:

- The FSA has commissioned an independent audit of the study, which will include sample collection, labelling, storage, handling, transport and analysis. Risk Solutions have been contracted to conduct this audit. It is anticipated that the audit will be completed and reported during October/November 2002.
- The FSA and VLA have agreed that the validation of the methods of analysis for this study should be extended to include samples of “soluble” PrPsc. Some preliminary work on the isolation of soluble PrPsc has

already been carried out under a DEFRA funded project (SE1762) and this will continue in collaboration with the NIBSC. It is the intention that soluble PrPsc resulting from that study will be used for the additional validation work.

It is anticipated the additional work will extend the method validation phase of the current study by about 3 months and this will in turn delay the reporting of the milk analysis results until early spring 2003.

Despite the delay the advantages are firstly, the validation data will be considerably more extensive and robust having included an analyte that is more likely to be in the form which may be present in the experimental milk samples. Secondly, the additional time scale will allow the results of the milk analysis to be provided on the first and second lactations.

- The VLA has been instructed to complete the “validation” of the analytical methods and submit the results for peer review by an independent team of expert analysts.

5. Future programme

Validation of the methods of analysis: The milestones for the additional method validation work are being revised by the VLA and it is now anticipated that this phase of the study will be completed during 31 October 2002 and reported to the FSA during November 2002. The methods of analysis and validation data will be submitted for peer review in December 2002 and the peer review report provided to the FSA early in 2003.

Analysis of experimental milk samples: The outcome of the study audit, and the peer review of the methods of analysis, including the validation data, will be used to decide the next stage of the study. Depending on the outcome, the VLA may or may not be authorised to proceed with the analysis of the experimental milk samples.

Milk sampling: The original research proposal did not envisage the collection of milk samples beyond the second lactation. The control and low dose group together with some (5) of the high dose group of cows, that have not shown

clinical signs of BSE, are about to enter a third lactation and milk samples will continue to be collected from these animals. The revised research proposal is included in ANNEX I (revision 09/08/02).

Milk samples are also being collected with the object of commissioning a milk bioassay study in the future. The protocol for this is the same as that requested by Prof Prusiner (ANNEX II).

Application Form for Research Project Financial Support From the Food Standards Agency

Proposal Full Title	DETERMINATION OF ABNORMAL PRION PROTEIN IN THE MILK OF CATTLE INFECTED WITH THE BSE AGENT
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- Applicants should complete each part of this form as fully and as clearly as possible
- This form should be completed in conjunction with the 'Form Completion Guidelines'

For Agency Use Only	
Proposal Code	M03016
Date Received	8 August 2002

PROPOSAL OVERVIEW

If the proposal is successful, information detailed in the Proposal Overview will be posted on the Agency Internet research web pages.

Full Project Title DETERMINATION OF ABNORMAL PRION PROTEIN IN THE MILK OF CATTLE INFECTED WITH THE BSE AGENT
Working Title PrP^{bse} in Milk

Project Lead Contractor

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Proposal Summary

There remains the possibility that milk produced by dairy cattle exposed to the BSE agent contains some infectivity itself and therefore poses a risk to the human population and to successive generations of cattle. The evidence for this possibility is derived mainly from interpretations of epidemiological data^{1,2,3,4,5} as no direct experimental data have been generated by appropriate methodologies to determine the presence of the agent in milk^{6,7}. This proposal seeks to provide that data by utilising the most sensitive PrP^{bse} detection procedures available and to link these analyses with subsequent infectivity bioassays on fractionated and concentrated milk from cattle in various stages of development of the disease. Milk is the only product derived from BSE-susceptible animals over thirty months of age which is available for public consumption and, as such, requires an analysis of the risk based on analytical data for effective policy control measures to be considered. The results will also provide direct experimental evidence for the involvement of milk in any maternal transfer of the disease and thus for MAFF policy for the control of BSE itself.

Duration (in months) 48 **Proposed Start Date** 01.09.00

Summary of total estimated costs (excluding VAT). Note: The Agency financial year is from 1 April to 31 March. Costs Should be recorded in line with this time scale.

Funding bodies	Financial Year 1	Financial Year 2	Financial Year 3	Financial Year 4	Financial Year 5	TOTAL (£)
Food Standards Agency						
Other than the Agency 'In kind'						
TOTAL COST (£)						

PART A – GENERAL PROPOSAL DETAILS

Full Project Title DETERMINATION OF ABNORMAL PRION PROTEIN IN THE MILK OF CATTLE INFECTED WITH THE BSE AGENT
Working Title PrP^{bse} in Milk

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Duration (in months) 48 **Proposed Start Date** 01.09.00

Will the research require a survey to be carried out, or a questionnaire to be used? YES or NO NO
Does any of the work outlined in the proposal require a licence from the Home Office under the Animal Scientific Procedures Act 1986? YES or NO YES

Summary of total estimated costs (excluding VAT).

Funding bodies	Financial Year 1	Financial Year 2	Financial Year 3	Financial Year 4	Financial Year 5	TOTAL (£)
Food Standards Agency						
Other than the Agency 'In kind'						
TOTAL COST (£)						

PART B – RELEVANCE TO THE FOOD STANDARDS AGENCY

Milk and milk products occupy a significant position in the human food chain, from early childhood to old age. In order to support its work on consumer protection, The Food Standards Agency needs to base its decisions, advice and policies on the best available science as funded through its research programme and as far as milk is concerned, the risk to the human population from animal transmissible spongiform encephalopathies remains undetermined. Indirect evidence from the epidemiology of maternal transfer in cattle^{1,2,3,4,5}, indicates a low risk, but direct analytical evidence^{6,7} for the presence of the infectious agent (as abnormal prion protein) is not yet fully available. This is particularly so with reference to large milk sample volumes and the possible presence of infectivity during the course of BSE. At the present time, milk is the only food source which continues to be obtained from animals developing BSE or even in terminal disease.

This proposal seeks to obtain and analyse up to 20 Litre samples of milk at timepoints spread over the final 2 years of disease in experimentally challenged animals. It will apply, initially, scaled up existing technology for PrP^{sc} detection to provide some interim data, whilst at the same time developing appropriate procedures for the detection of low concentrations of agent in large volumes of milk. In this way, data for an effective risk assessment for bovine milk will be provided to aid in the management of that risk for the human population.

PART C – DESCRIPTION OF SCIENTIFIC / TECHNOLOGICAL OBJECTIVES, WORKPLAN AND FINANCES

(Details declared here will go to form ‘Section 3 - Scope of Work’ of the research contract if the proposal is successful)

C1. Objectives and Expected Achievements

Objective No.	Objective Description
01	Source and secure milk samples from BSE-challenged cattle and the equipment, facilities and resources to deliver the samples
02	Develop and agree milk fractionation, processing and storage procedures. Agree sample collection size and frequency.
03	Collect, process and store milk and milk fractions according to agreed timetable and procedures (Aug 2000 – November 2003)
04	Develop and validate PrP determination procedures for the somatic cell fraction of milk
05	Analyse all available cell fraction milk samples (from colostrum to 40 weeks post calving)
06	Assess ability of existing polyclonal and monoclonal antibodies to bind non-denatured PrPc and PrPbse in milk (+/- PrPbse spike) by solid phase immunoaffinity extraction followed by detection on SDS-PAGE Western blots.
07	Review project progress and analytical methodology. Consider and agree any requirement for bioassay of milk fractions and preparations for submission of a separate proposal to address this area.
08	Produce phage display library and select PrPbse – binding clones
09	Apply affinity maturation procedures, re-clone and produce high affinity antibody fragments
10	Apply ultracentrifugation procedures, currently used for PrPbse concentration from brain homogenates, to homogenised / pasteurised whole milk
11	Develop and produce solid phase immunoaffinity extraction matrices from antibody(s) indicated in 06 above. Determine recovery, reproducibility, selectivity and limit of determination of the analyte from whole milk.
12	Review project progress and analytical methodology. Determine and agree protocols to pursue for the analysis of experimental milk samples
13	Develop and produce solid phase immunoaffinity extraction matrices from antibody(s) produced in 09 above. Determine recovery, reproducibility, selectivity and limit of determination of the analyte from whole milk.
14	Validate targeted analytical process and apply to milk samples
15	Submit final report and draft publication(s)

C2. Approaches and Research Plan

Objective 01 (A flow chart for the project is inserted at Appendix A)

It has been necessary to achieve objective 01 in advance of the acceptance of this proposal due to the constraints of the BSE challenge of cattle experiment (SE1736) essential for the supply of milk samples from cows orally exposed to the BSE agent and developing disease. All animals were challenged in 1998 at around 3 months of age and the experiment was designed for the periodic slaughter and archiving of a wide range of tissues and body fluids throughout the course of the disease. Following the SEAC / MAFF call for the need to study milk for the presence of the BSE agent, 10 animals from the high-dose challenged group (100g BSE infected brain material), 10 animals from the low-dose group (1.0g challenge) and 10 non-exposed controls were diverted from the existing project, artificially inseminated and calved between August and December 2000. This timetable was necessary to produce the first lactation as early as possible - 2 years after challenge – and to ensure at least one lactation cycle before clinical disease. As a result, animal handling and milking facilities have been set up at the establishment undertaking SE1736 and appropriate excess milk decontamination and disposal facilities provided, all costs and resources being covered within that project. It has been the responsibility of Participant 01 to ensure delivery of milk within the timetable, agreement of milking and processing protocols to ensure integrity of samples and elimination of cross-contamination, installation and use of milk processing equipment and transportation and storage of samples.

Task 1. (P.01). Install protocols for the collection and processing of milk from experimental animals. Draw up husbandry protocol and sampling timetable. 1.5 pm. 01.09.00 – 01.10.00. To enable collection of milk samples

Task 2. (P.01) Source and install equipment for milk processing and storage. Commission and draw up protocols for use and to assess throughputs. 1.5 pm. 01.09.00 – 15.10.00. To enable on-site processing of milk and subsequent storage. Draft protocols for the milk collection and processing are appended at Appendices B & C.

Objective 02

Guidance and FSA requirements for sample collection size and frequency, sample processing and fractionation and eventual storage of materials have been given at a meeting on 7th August between FSA, TSERSU, VLA and advisors and in subsequent correspondence (appendices D & E). As far as possible, these requirements have been and will be met, except where logistical and resource constraints are limiting.

Experimental animals: 30 lactating cows: Group B2, 10 orally challenged with 100g of BSE-affected brain as a 10% homogenate, Group B3, 10 orally challenged with 1g BSE-affected brain as a 10% homogenate and Group B1, 10 unexposed controls. Calving dates range from the end August 2000 to an expected early December 2000. All challenges were carried out during September 1998. Clinical disease is expected within a window of 32 – 74 months post-challenge, but this is based on data from relatively few transmission studies.

Sample size: At each individual timepoint a maximum sample size of 20 litres will be taken as a representative subsample of the total milking. During early and late lactation when yield will be lower, a combined milk sample from 2 milkings in a single 24-hour period up to a representative 20 litres will be taken.

Sampling frequency: There will be 5 sampling timepoints during each lactation; (1) Within 7 days of calving (colostrum). (2) at 10 weeks, (3) At 20 weeks, (4) At 30 weeks and (5) at 40 weeks). Within each sampling timepoint, there will be 3 collections spread over a 7-day period for subsequent collection of the somatic cell fraction. It is not possible to stipulate specific days during each 7 day timepoint because of the throughput restrictions on the milking parlour due to decontamination of equipment between samplings and the need to accommodate weekend working for milk processing and transport. For the collection of whole milk (pasteurised and homogenised) a single 20 litre sample will be collected at each time point.

Animal numbers: For the collection of whole milk, all 30 animals will be sampled as above. For the preparation of somatic cell fractions, separate 10 litre representative subsamples will be taken from one milking from 4 or more animals in each group (A, B & C). As far as possible this will be the same 4 animals sampled throughout the study. This restriction is necessitated by transport, centrifugation and processing throughput constraints. Even so, this involves the processing and collection of somatic cell fractions from 360 Litres of milk within each 7-day timepoint. Previous definitive pathogenesis and tissue infectivity studies of BSE in cattle also utilised a maximum of 4 animals per Group.

A major factor in the timescales involved will be the success rate of the artificial insemination take-up rate. 100% is never achieved and so some animals will require re-insemination up to 5 months after the target date.

Intercurrent disease: Within any dairy herd, mastitis is likely to occur in some animals at some time during the lactation cycle. This will be treated with antibiotics and standard husbandry considerations as required. Occasionally, this may affect milk yields at particular sampling points during which time total 24 hour samples will be collected. Any animals suffering mastitis between designated sampling timepoints will supply additional samples from affected udders as and when available.

Task 1. (P.01) Trials and resource estimates on sample collection and processing. 1.5 pm. 15.10.00 – 01.11.00. To allow sampling throughput and timetable to be constructed.

Task 2. (P.01) Determine maximum throughput and sampling timetables. Agree with FSA. 0.5 pm. 01.11.00 – 15.11.00. To instigate an agreed sampling and processing procedure.

Objective 03

Collection, processing and storage of milk and milk fractions started in August 2000 and will continue until approximately November 2003. After the first lactation cycle, the animals will be dried off and re-inseminated to calve during the autumn of 2001 and the milk collection and processing sequence will be repeated. Not all animals will be successfully inseminated at the first attempt and this will lead to some staggering of the lactation cycles between animals.

Task 1. (P.01) Collect, process and store somatic cell fractions according to agreed frequency and timetable from first lactation cycle. 7 pm. 15.09.00 – 01.07.01. To collect cell fraction samples.

Task 2. (P.01) Collect, pasteurise, homogenise and store whole milk from all experimental animals at 1 week, 10, 20 and 30 weeks post calving during the first lactation cycle. 4 pm. 15.09.00 – 01.07.01. To collect whole milk samples

Task 3. (P.01) As Task 1 for the second lactation cycle. 8 pm. 01.09.01 – 01.09.02.

Task 4. (P.01) As Task 2 for the second lactation cycle. 4 pm. 01.09.01 – 01.09.02

5. (P.01) As Task 1 for the third lactation cycle. 9 pm. 01.09.02 – 01.11.03

(P.01) As Task 2 for the third lactation cycle. 4 pm. 01.09.02 – 01.11.03

Task
Task 6.

Objective 04

Procedures will be developed for the analysis of the somatic cell fraction for (initially) PrPc and then for PrPsc. These will be based on traditional, validated procedures for the determination of PrP in brainstem material and applied to the pellet of somatic cells obtained from the low speed centrifugation of the milk. Three procedures will be assessed for the greatest analytical sensitivity for the matrix within acceptable reproducibility. (1) The Prionics procedure – a standardised, denaturing polyacrylamide Gel Electrophoresis separation of proteinase K resistant prion protein, visualised by Western blot and probing with 6H4 anti-PrP monoclonal antibody. This procedure was successful in the DGXXIV EU Evaluation Exercise for disease associated prion detection in bovine brainstem. (2) The CEA / Biorad procedure – a standardised, commercially available, extraction procedure for proteinase K resistant denatured PrPbse followed by a sandwich enzyme immunoassay, also successful in the EU Evaluation exercise. (3) The DELFIA procedure – a standardised guanidine solubilisation of abnormal PrP (no proteinase K digestion) followed by a sandwich fluoroimmunoassay and which has been submitted for the second EU Evaluation Exercise due November 2000. The primary determinants will be the elimination of any milk matrix effects on the endpoint assay by extract dilution and the ability of the procedures to detect disease associated PrPbse in a (likely) non-aggregated form. Careful titration of proteinase K (Procedures 1 & 2), evaluation of centrifuge speeds (Procedures 2 & 3) and guanidine concentrations (Procedure 3) will be necessary to determine recoveries of endogenous total PrP, spiked recombinant PrP and spiked extracts (centrifuged and non-centrifuged BSE brainstem) from milk cell fractions.

Following development of the procedures for their application to milk cell fractions, an extensive validation process will be undertaken on any of the analytical test(s) chosen as suitable. This will occur in 2 phases, the first utilising analytical spike material of BSE-brain homogenate and the second utilising a “low aggregate” state of PrPbse, more representative of the likely form of prion that may be present in milk. This latter spike material will require development and characterisation prior to use. Both validation studies will be submitted for external peer review, which will report prior to any analysis of experimental milk samples. At the same time an external audit of the sample collection, transport, processing and storage procedures will also be undertaken.

Task 1. (P.01) Assessment of the application for milk somatic cells as an analytical matrix in the Prionics test for PrPbse. Determination of maximum sample size and recovery of spiked material. Development of procedures for increasing sample size where necessary, titration of proteinase K reagent to ensure degradation of PrPc with minimal loss of PrPbse. Validate procedure using BSE-affected brain spikes. 10 pm. 01.11.00 – 01.02.03. To produce a validated PAGE / Western blot assay for PrPbse in milk cell fractions.

Task 2. (P.01) Assessment of the application for milk somatic cells as an analytical matrix in the CEA / Biorad test for PrPbse. Determination of maximum sample size and recovery of spiked material. Development of procedures for increasing sample size where necessary, titration of proteinase K reagent and optimisation of centrifugation speeds to ensure elimination of PrPc with maximal recovery of PrPbse. Validate procedure using BSE-affected brain spikes. 10 pm. 01.02.01 – 01.11.02. To produce a validated ELISA procedure for PrPbse in milk somatic cells.

Task 3. (P.01) Assessment of the application for milk somatic cells as an analytical matrix in the DELFIA test for PrPbse (which does not require Proteinase K digestion). Determination of maximal sample size and recovery of spiked material. Development of procedures for increasing sample size where necessary, titration of guanidine extraction and optimisation of centrifuge speeds to ensure elimination of PrPc with maximal recovery of PrPbse. Validate procedure.

4 pm. 01.01.01 – 01.06.01. To produce a validated immunoassay without the need for proteinase K digestion for PrP^{Sc} in milk somatic cells.

Task 4. (P.01) To select the most appropriate test from above for application to experimental samples.

Task 5. (P.01) To develop a “low-aggregate” preparation of PrP^{Sc} and characterise 4pm 01.08.02 – 01.11.02.

Task 6. (P.01) To validate ELISA and Western Blot tests utilising “low-aggregate spikes. 4 pm 01.11.02 – 15.02.03

Objective 05

On selection and validation of the most appropriate analytical procedure for the somatic cell fractions (end March 2003), all samples already archived will be analysed and will be reported as a batch of results. This will include most of the samples from the first and second lactation cycle. Subsequent analyses will be carried out on an ongoing basis as samples are collected until November 2003 with analyses completed by March 2004.

Task 1. (P.01) To analyse all somatic cell samples collected during the first lactation cycle for PrP^{Sc} and to report to FSA.

2 pm. 01.03.03 – 01.04.03. To report results of analyses for PrP^{Sc} in milk somatic cell samples.

Task 2.

(P.01) To analyse all somatic cell samples collected during the second lactation cycle for PrP^{Sc} and to report to FSA. 3

pm. 01.04.03 – 01.05.03

Task 3. To analyse all somatic cell samples collected during the third lactation cycle for PrP^{Sc} and to report to FSA. 3

pm. 01.01.04 – 01.03.04

Objective 6

A range of existing polyclonal and monoclonal antibodies, available either from VLA or from other Laboratories, will be selected for assessment as immunoaffinity extraction reagents. Whilst there have been no reports of any current antibodies which possess the capability of binding to PrP^{Sc} in its non-denatured form, few have been properly evaluated for immunoaffinity extraction potential. However, it must be stressed that objective 6 is an evaluation exercise which makes no claim to be able to source an appropriate immunoreagent, but will eliminate the pre-existence of antibodies to PrP^{Sc}. Nevertheless, selection of antibodies will be made to provide the greatest chance of success. To this end, PrP-peptide antibodies, raised to the C and N termini of the PrP molecule (and arguably most likely to bind to the altered form of PrP) as well as those raised to intermediate points will be included. In addition, antibodies to disease specific PrP from cattle, sheep, mouse and hamster will be assessed. As well as the necessity for a potential PrP^{Sc} recognition, any antibody assessed must be available in sufficient quantities for all studies in this proposal and for future possible diagnostic use. It is likely that about 10 antibodies can be exhaustively assessed in the time period

The assessment technology will be based on a magnetic bead immunoaffinity extraction system. Each candidate will be immobilised on the beads and used to extract a standardised BSE brain pool homogenate and milk spiked with that homogenate. Each will be assessed before and after proteinase K digestion. Comparison will be made with negative brain and with whole, control milk. Following immunoaffinity extraction, the beads will be subjected to the standard denaturing buffer (SDS) prior to PAGE / Western blot analysis of any extracted PrP.

Task 1. (P.01) Apply existing magnetic bead immunoaffinity extraction process to the screening of candidate antibodies for PrP^{Sc}. Develop procedures for the immunoaffinity extraction of (1) PrP^{Sc} following Proteinase K digestion of the sample and of (2) PrP^{Sc} prior to Proteinase K digestion and of (3) total PrP with no Proteinase K digestion, and assess on Western blots. 4 pm. 01.01.01 – 01.04.01. To produce a protocol for the assessment of antibodies to extract PrP^{Sc} from milk.

Task 2. (P.01) Screen candidate antibodies and select any showing significant binding to PrP^{Sc} (30 – 33 or 27 – 30). 3 pm. 01.04.01 – 01.07.01. To identify any existing antibodies capable of the immunoaffinity extraction of PrP^{Sc} from milk.

Objective 7

It is pertinent to set a timetable of Review meetings between the Customer and Contractors for the course of the project, at 6-monthly intervals from the time of acceptance of the proposal. It is suggested that these be held at the end of February and August each year. As well as satisfying the Customer of progress, there are a number of points within the project where the direction of the project will require discussion and confirmation. Primarily these will occur following (1) completion of the first batch of somatic cell fraction analyses in Objective 05 whereby a positive finding of disease associated PrP will signal the further development and use of the procedures involved, (2) at the completion of Objective 06 whereby the sourcing of a PrP^{Sc} – binding existing antibody will preclude the necessity for continuation of Objectives 08, 09 & 13. Conversely, failure to find such an antibody in Objective 06 will preclude Objective 11 but highlight further the necessity for Objectives 08, 09 & 13. A further important consideration to be made at the end of the first year will be the need for undertaking bioassays for infectivity of samples and extracts. These will be essential if putative PrP^{Sc} is detected in any of the procedures employed but will depend on the likelihood of a retention of infectivity in those extracts. Should bioassays be indicated, a proposal – separate to or as an addition to this proposal – will be required.

Task 1. (P.01 / P.02 / FSA) To hold Project Review Meetings in February and August 2001 and February 2002. 0.5 pm

Objective 08

Targeted donor animals from 06 above and from other MAFF projects (SE1761, SE1731, SE1728) above which are still alive (mainly sheep), will be boosted, and spleens taken for lymphocyte extraction. If rabbit or rat antisera is targeted, new animals will be immunised and boosted with PrP- immunogen, and spleen lymphocytes prepared. With the target of improving affinity by 10^3 , spleen cells of sheep or rabbit in which high-avidity antibodies have been raised against PrP or PrP-peptides will be used as a source of antibody gene sequences for recombinant antibody construction. For each spleen, total RNA will be isolated using commercial kits and used as a template for first-strand cDNA synthesis, employing random hexanucleotide primers. The first-strand cDNA will then be used as template for PCR amplification of V gene sequences (VH and VL). Degenerate primers, derived from available sheep or rabbit V gene sequence databases, will be used in the amplification. The PCR products will be restricted and ligated into the appropriate sites in a phage display vector (pSD3). These ligations will link the two variable domains via a polypeptide linker sequence, creating a single-chain Fv gene. The recombinant phagemid will be electroporated into TG1 host cells, to create an scFv library. After library amplification, phage-displaying scFvs would be produced by rescue with VCSM13 helper phage. The displaying phages will then be affinity selected against the relevant antigens in order to isolate those clones displaying functional antibodies. This selection process will be repeated several times in order to achieve the selection of high-affinity antibody fragments. Individual binders will be cloned and sequenced. The selected recombinant antibodies will be expressed in a bacterial host, purified and used for characterisation of specificity and affinity.

Task 1. (P.01). To immunise and boost donor animals with PrP-peptide and PrPbse immunogens. Assessment of antisera for PrPbse (denatured) binding potential to ensure immune response. 1 pm. 01.08.00 – 01.12.00. To select animals for spleen lymphocyte collection.

Task 2. (P.02) To develop and optimise phage display library and vector (pSD3). To collect total RNA for first strand cDNA synthesis, amplification of VH and VL gene sequences by PCR and ligation into phage display vector. Electroporation of the recombinant phagemid into TG1 host cells to create an scFv library and subsequently amplify. 10.5 pm 01.07.00 - 01.04.01. To create and amplify scFv library.

Task 3. (P.02) To produce phage-displaying scFvs by rescue with VCSM13 helper phage and to affinity select those clones showing appropriate binding to non-denatured PrPbse antigen. To clone and sequence positive binders and express their recombinant antibodies in a bacterial host. 3 pm 01.04.01 – 31.09.01. To express antibodies.

Task 4. (P.02 and P.01) To purify and characterise recombinant antibodies for specificity and affinity for PrPbse. 2.5 pm (P.01), 2.0 pm (P.02). 01.09.01 - 01.08.02. To characterise antibodies.

Objective 9

If the affinities of isolated antibody fragments do not approach the required level, mutations will be introduced to the antibody combining sites of isolated fragments. Different methods, such as random mutation or site-directed mutagenesis, could be applied either alone or in combination. Mutated antibody fragment gene fragments will be re-cloned in to the pSD3 vector again. Antibody fragments with improved affinity will be isolated following a modified re-selection against the same PrP-peptide. The approach is repeated until the antibodies with required affinity have been isolated. Sufficient quantities of antibody will then be prepared following re-cloning in to the pSD3 vector.

Task 1. (P.02) To Introduce mutations using random methods, into gene sequences encoding selected antibody fragments, clone into pSD3 vector and display the mutated antibody fragments for affinity selection. Error-prone PCR will be used for introduce random modifications into the selected antibody genes. The mutated antibody genes will be re-amplified, digested and ligated into pSD3 to create sub-libraries. 2 pm 01.08.02 - 01.11.02. To create sub-libraries.

Task 2. (P.02) The sub-libraries will be used biopanning against surface-immobilised prion proteins. Selection methods will be designed so as to bias the selection towards the isolation of higher affinity antibodies. The following modifications of selection methods will be employed: 1. Reducing target antigen concentration. 2. Reducing antibody-antigen interaction time, favouring the selection of antibodies with faster on-rates. 3. Increasing washing time, favouring the selection of antibodies with slower off-rates. 6 pm. 01.04.02 - 01.08.02. To recover first batch antibodies.

Task 3. (P.02) To purify and characterise recombinant antibodies for specificity and affinity for PrPbse. 3 pm. 01.08.02 – 01.12.02. To characterise first batch antibodies.

Task 4. (P.02) To repeat tasks 1 - 3 until required affinity is obtained and harvest large-scale production of the new antibodies. 7 pm 01.12.02 - 01 - 01.03.03. To produce antibodies.

Objective 10

This is a part of the study similar in principal to Objective 04 in that it is a selective process to enable the possibility of finding a positive result earlier in the project. Whilst the immunoaffinity extraction of the whole milk utilising antibodies designed and produced for that purpose, remains by far the best means of concentrating and purifying the analyte prior to detection, ultracentrifugation is a relatively simple but highly resource dependent means of concentrating PrPbse. Most

analytical methods for brain PrP utilise a centrifugation step but this relies on the protein being insoluble and, primarily, aggregated. There is no direct indication as to whether PrP^{Sc} is present at all in milk but if it does occur it is most likely to be at a very low concentration and in a non-aggregated form. This may still be insoluble enough to respond to high-speed ultracentrifugation which will be carried out, necessarily, on small aliquots of the whole milk collected. It is noted that the CEA / Biorad test kit employs a "precipitation" buffer to improve recovery of PrP^{Sc} at lower g-forces from brain Homogenates and this will also be assessed for the treatment of milk. In order to assess and provide some validation of the centrifugation techniques, milk spiked with BSE brain homogenate will be used with the proviso that such a spike will contain considerable quantities of aggregated PrP^{Sc} which is not expected to be present in the milk samples. Pellets of material supplied by ultracentrifugation will be analysed by the 3 PrP detection procedures described in Objective 04.

Task 1. (P.01) To prepare pellets of insoluble material from whole milk by centrifugation and apply to PrP detection assays to determine maximal sample size, matrix effects and recovery. 3.5 pm 01.07.01 – 01.11.01. To apply ultracentrifugation as a means of preparing milk extracts for PrP^{Sc} detection.

Task 2. (P.01) To apply above methodology to experimental milk samples. 4 pm 01.11.01 – 01.06.02

Objective 11

This objective depends entirely on whether any pre-existing antibodies can be utilised in immunoaffinity extraction format for the concentration of abnormal PrP from milk as determined in Objective 06 above. As soon as one (or more) may be identified, a new timetable for the project will be negotiated with FSA, to allow Objective 11 to proceed at the earliest possible date. This will involve the purchase or preparation of large quantities of the targeted antibody and development of scaled up procedures for the immunoaffinity extraction of 10 – 20 Litres of homogenised milk. Whilst antibody coated magnetic beads will be used as the ideal solid phase in the selection process, it is considered that efficiency will be compromised on scale up and that an appropriate immobilisation matrix for use in a column extraction format will be required for large quantities of milk. Preferred solid phase matrices will be based on porous glass or silica, ie non-compressible materials, selected for their antibody immobilisation capacities, inertness to sample non-specific absorption and capacity for large volume sample passage. Size of immunoaffinity column will be dictated by prion protein binding capacity which will be required to be in excess of the total "normal" PrP^C present in the milk sample. At this stage of the project it is unlikely that any antibodies will be available which have a preferred binding to abnormal PrP^{Sc} but relative PrP isoform affinities will be an important consideration. The large scale antibody production will be carried out by either P.01 for polyclonal(s) – for which a special Home Office Project Licence will be required - or by a within-laboratory sub-contract to the Cell Technology and Culture Unit who specialise in the production and purification of monoclonal antibodies from archived cell lines.

Columns will be assessed using, initially, model labelled proteins such as tagged recombinant PrP (Prof S Weiss, University of Munich) or PrP-peptide conjugates for their efficiency, reproducibility and recovery. In addition, scale up efficiency of the extraction from milk from low to high volumes will be calculated. Compatibility of the immunoaffinity-extracted sample with endpoint assays will also need to be ensured. Development of antigen elution techniques based on the original bead technology such as treatment with denaturing or chaotropic solutions such as sodium dodecyl sulphate or guanidinium hydrochloride will also be carried out – particularly prior to Western blot analysis. More selective eluting solutions such as glycine or low / high pH may be more selective and produce an analytically cleaner extract. Finally, recovery of abnormal PrP from milk spiked with BSE-affected brain homogenate will be carried out, but with the reservation that the PrP^{Sc} in brain will not (or may not) be present in the same physical form or structure as that which may be present in the milk of animals suffering from BSE.

Task 1. (P.01) Purchase or preparation of antibody supplies 4 pm 01.07.01 - 01.11.01. To supply antibodies for IAE

Task 2. (P.01). Large model system + assessment and characterisation. 9 pm 01.04.01 – 01.01.02 To develop large scale immunoaffinity extraction process.

Task 3. (P.01). Prepare columns with targeted anti-PrP antibodies. 5 person months 01.01.02 – 01.01.02. To prepare large scale prion protein extraction process.

Objective 12

With experimental data available for the possible existence of antibodies capable of recognising abnormal PrP and the format for the immunoaffinity extraction of large volumes of milk determined, a review of the production of new antibodies by Collaborator 02 will be required. Protocols for the analysis of experimental milk samples will be agreed.

Task 1. (P.01 / P.02 / FSA). Hold Fourth and 5th Project Review. 01.08.02, 31.03.03

Task 2. (P.01 / P.02 / FSA) Hold Final Project Review. 01.09.03

Objective 13

This objective is essentially the same as Objective 11 but has a different time frame and follows on directly from Objective 09. It is the author's considered view that development of new antibodies capable of binding to abnormal PrP in non-denatured, large volume solutions are essential for the achievement of the primary objective of this proposal – the detection of low level PrPbse in milk. Immunoaffinity columns utilising the best of the new antibodies will be prepared and assessed as above in Objective 11. However, unless a linear epitope can be identified and an equivalent peptide sourced to produce a synthetic antigen, recovery assessment will be made from titrations of PrPbse spike material. Because of the expected high affinities of new antibodies, elution strategies will also need to be developed.

Task 1. (P.01) Prepare large-scale immunoaffinity columns with new antibodies. Undertake matrix effects and non-specific binding effects, 6 pm 01.03.03 – 01.08.03

Task 2. (P.01) Assess and characterise columns for PrPbse recovery from milk. 3 pm 01.08.03 – 01.10.03 **Objective 14**

The immunoaffinity extraction process and the subsequent quantification of PrPbse will be subjected to the exhaustive validation procedures developed in Obj. 04. Utilising spikes from both BSE-affected brain homogenate and "low aggregate" preparations of PrPbse. The results of the validation exercise will be submitted to FSA for external peer review. No analysis of experimental whole milk samples will be attempted until agreement is reached with FSA on the applicability of the procedures.

Task 1 (P.01) Undertake a full validation study for the immunoaffinity extraction and detection of PrPbse from milk utilising spikes of BSE-affected brain and "low aggregate" PrPbse as in Obj. 04. Submit validation data to FSA for external peer review prior to application to experimental milk samples. 5 pm 01.08.03 – 01.12.03. **Task 2. (P.01)**

Analyse 1st, 2nd and 3rd lactation cycle whole milk samples and report to FSA. 8 pm 01.12.03 – 01.09.04 **Objective 15**

Interim reports will be produced annually as required in the contract as well as additional reports for the regular Review Meetings. The final Project Report will be submitted by 30.09.2004.

It is expected that papers for submission to peer reviewed publications will result from the study of the somatic cell fraction, the development of immunoaffinity protocols (showing PrPc) for milk, the development and characterisation of antibodies and the final milk analyses.

Task 1.
(P.01, P.02,) To submit to FSA interim and annual reports to deadlines for interim reviews and for annual and final project reports. 2.0 pm Throughout the life of the project

Task 2.
(P.01, P.02,) To submit through FSA to peer reviewed journals, papers on technology development and of analytical results. Expected output 4 publications 3.5 pm.

C3. Project Milestones

Milestone	Target Date	Milestone Title
01/01	01.10.00. month 1	Source and secure milk samples from BSE-challenged cattle
01/02	01.10.01 m 1	Set up and install milking equipment and processing facilities at Drayton
02/01	15.10.01. m 1	Initiate milk collection and processing trials
02/02	15.11.00. m 2	Agree sample collection size and frequency and sample processing with FSA
03/01	01.09.01. m 12	Collect, process and store milk from experimental animals during first lactation
03/02	01.10.01. m 12	Dry off, serve and calve experimental cattle

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03/03	01.09.02. m 24	Collect, process and store milk from experimental animals during second lactation
03/04	01.11.03. m 38	Collect, process and store milk from experimental animals during third lactation
04/01	01.08.01. m 11	Apply and assess control milk somatic cell fractions to PAGE / Western blot, Prionics, Bio-Rad and DELFIA assays
04/02.01	30.09.02. m 24	Develop or modify sample extraction procedures including titration of Proteinase K and validate procedures on BSE-brain spiked samples
04.02.02	15.10.02 m26	Submit validation data to FSA for external peer review
04.02.03	01.11.02 m26	Submit to external audit of sample management processes
04.02.04	01.12.02 m27	Develop "low-aggregate" PrPbse spike
04.02.05	15.02.03 m30	Complete validation exercise using "low-aggregate" spike
04.02.06	01.03.03 m30	Submit validation data to FSA for external peer review
05/01	01.05.03. m 31	Apply to somatic cell fractions collected during first lactation cycle
05/02	01.06.03. m 32	Apply to somatic cell fractions collected during second lactation cycle
05/03	01.03.04	Apply to somatic cell fractions collected during third lactation cycle
06/01	01.12.01. m 15	Assess a range of existing antibodies for the extraction of PrPbse
07/01	01.08.01. m11	Hold First Project Review. Report on somatic cell fraction analysis, initial antibody assessment and new antibody progress
07/02	01.01.02. m16	Hold Second Project Review. Completed cell fraction analysis and antibody assessment, new antibody progress and need for bioassays
07/03	01.07.02. m 22	Hold Third Project Review. Immunoaffinity extraction, new antibody progress
08/01	01.03.02. m 18	Produce phage display antibodies.
08/02	01.08.02. m 23	Determine specificities and affinities of phage display antibodies
09/01	01.11.02. m 26	Generate mutations to increase antibody affinity
09/02	01.03.03. m 30	Harvest large scale production of new antibodies
10/01	01.11.01. m 14	Apply ultracentrifugation as a means of concentrating PrPbse from milk. Validate processes as appropriate
10/02	01.06.02. m 26	Apply ultracentrifugation to experimental milk samples – if appropriate
11/01	01.10.02. m 25	Produce immunoaffinity support(s) from existing antibody(s). Determine recovery, selectivity, reproducibility and limit of determination
12/01	01.01.03. m 28	Hold Fourth Project Review. Methodology review and agreement of analytical processes.
12/02	31.08.03. m 36	Hold Fifth Project Review. Results, reports and publications

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13/01	01.10.03. m 37	Produce immunoaffinity support(s) from new antibody(s). Determine recovery, selectivity, reproducibility and limits of determination
14/01	01.12.03. m 39	Complete validation of targeted analytical procedures for whole milk samples
14/02	01.09.04. m 48	Apply validated procedures to whole milk samples from experimental animals
15/01	30.09.04. m 49	Hold Final Project Review. Submit final project report and draft publications to FSA

C4. Project Deliverables

Deliverable	Target Date	Deliverable Title
01	01.08.01. m 11	Report and First Review Meeting
02	01.09.01. m 12	Milk and milk fractions from BSE-challenged cattle (First Lactation)
03	01.09.02. m 24	Milk and milk fractions from BSE-challenged cattle (Second Lactation)
04	01.09.03 m 36	Milk and milk fractions from BSE-challenged cattle (Third Lactation)
05	01.11.02. m 26	Validation of tests (using BSE-brain spikes)
06	01.03.03 m 30	Validation of tests (using “low-aggregate” spikes)
07	01.05.03. m 32	Results from the analysis of somatic cell fractions (First & Second Lactation) of milk cell fractions for PrPbse
08	01.01.02. m 16	Report and Second Review Meeting (Cell fraction analysis, assessment of currently available antibodies for immunoaffinity extraction, new antibody progress, need for bioassays)
09	01.07.02. m 22	Report and Third Review Meeting (Immunoaffinity extraction progress, new antibody production progress)
10	01.11.02. m 26	Assessment of the suitability of ultracentrifugation for the concentration of PrPbse from milk and, if appropriate, its use for the analysis of whole milk
11	01.03.03. m 30	Production of high affinity, PrPbse – recognising phage display antibodies
12	01.01.03. m 28	Report and Fourth Review Meeting (Methodology review, new antibody production review and agreement to proceed)
13	01.12.03. m 39	Development and validation of solid phase immunoaffinity extraction columns and targeted analytical procedures
14	01.09.04. m 48	Results from the analysis of whole milk for PrPbse

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01.10.04. m 49

Final Report and Review Meeting

C5. Role of Participants

Participant 01. Lead Contractor

Objectives:

1. To secure, process and store milk samples from experimental animals. Tasks: Install equipment, resources and protocols. Staff: 3 person months, year 1
2. To produce and agree sample collection size, frequency, processing, fractionation and storage protocols and timetable. Tasks: Undertake trials and resource estimates for milk samples. Agree protocols with FSA. Staff 2 pm, Yr 1.
3. To collect, process and store somatic cell fractions and whole milk samples from experimental animals from 3 lactation cycles. Tasks: Collect milk samples as per timetable, centrifuge to isolate somatic cells and store. Staff 11 pm, Year 1; 12 pm Year 2; 13 pm Years 3 and 4.
4. To develop procedures for the determination of PrP^{bse} in somatic cells. Tasks: Assess application of the Prionics, CEA/Biorad and DELFIA endpoint detection systems to the analysis of milk somatic cell fractions. Tasks: Determine sample application size and proteinase K treatment conditions and optimise for milk somatic cell fractions by the Prionics test. Determine sample application size, proteinase K treatment conditions and centrifugation parameters and optimise for milk somatic cell fractions by the CEA / Biorad test. Determine sample application size, guanidine concentration profiles and centrifugation parameters and optimise for milk somatic cell fractions by the DELFIA test. Staff, 7 pm, Yr 1; 5 pm Yr 2. To undertake full validation of appropriate methods and submit to external peer review. 8pm Yr 2, 12pm Yr 3.
5. To apply the most appropriate protocol from 4 above to the experimental milk samples. Tasks: Analyse somatic cell fractions and report results. Staff: 2 pm, Year 3 for 1st lactation; 2 pm Year 3 for 2nd lactation and 3pm Year 4 for 3rd lactation
6. To assess a range of existing anti-PrP antibodies for their ability to bind to abnormal PrP in its non-denatured form for use in immunoaffinity extraction of milk. Tasks: Select up to 10 antibodies, available in large quantities, and use in a magnetic bead immobilisation system for the immunoprecipitation of PrP^{bse}. Detect products by PAGE / Western blot. Staff: 4 pm, Year 1; 3 pm Year 2.
7. To set up and undertake 6 monthly Project Review Meetings between Customer and Contractors. 0.15 pm Year 1; 0.35 pm Year 2.
8. To prepare spleens from immunised animals and characterise antibodies. 1.0 pm, Year 1. 2.5 pm Year 2.
10. To apply ultracentrifugation for concentrating PrP^{bse} from milk. Tasks: Determine sample application size, matrix effects and recovery of PrP^{bse} by ultracentrifugation. If agreed, apply to whole milk. Staff: 4.5pm, Yr 2; 3.0pm Yr 3.
11. To purchase / produce (existing) antibodies for immunoaffinity columns and to develop and characterise large capacity columns. 16 pm, Year 2; 2 pm Year 3.
12. To set up and undertake further 6 monthly Project Review Meetings between Customer and Contractors. Staff: 0.45 pm Year 3
13. To assess recovery of PrP^{bse} from large-scale immunoaffinity extraction columns utilising new antibodies prepared by P.02. Tasks: Utilise pre-validated large scale immunoaffinity extraction columns from Obj 11 to assess efficiency of PrP^{bse} recovery from milk. Staff: 8 pm, Year 3, 1pm Year 4
14. To validate procedures to be used for the determination of PrP^{bse} in milk and apply them to experimental samples. Tasks: In consultation with FSA QAU undertake validation studies and provide validation document(s) prior to application to experimental milk samples. Staff: 5 pm, Year 3; 8 pm Year 4
15. To produce interim and final reports and papers for submission to peer reviewed journals. Staff: 0.25 pm Year 1; 1.5 pm Year 2; 1.5 pm Year 3; 2.25 Year 4.

Participant 02 ADAS / University of Leicester

Objectives:

7. To participate in 6 monthly Project Review meetings between customer and contractors. 0.05 pm Yr 1, 0.1 pm Yr 2.
8. To produce phage display library, select PrP^{bse} – binding clones and characterise recombinant antibodies. Tasks: To create and amplify scFv library. To select positive binding clones and to express antibodies in a bacterial host. Staff: 11.5 pm Year 1, Tasks: To purify and characterise antibodies. Staff: 4 pm Year 2.
9. To produce high affinity PrP^{bse} recombinant antibodies. Tasks: To introduce mutations and create sublibraries. To recover and characterise first batch antibodies. Staff: 12 pm Year 2, Tasks: To repeat Year 2 tasks until required affinities obtained and to harvest antibodies. Staff: 6 pm Year 3.
12. To undertake further 6 monthly Project Review Meetings between Customer and Contractors. Staff: 0.2 pm Year 3
15. To produce interim and final reports and papers for submission to peer reviewed journals. 0.25 pm, Year 1; 0.5 pm Year 2, 1 pm Year 3

C6. Project Management

The lead contractor, P.01, will chair the project management group and will be responsible for the delivery of the project milestones. The PMG will meet regularly at 3 monthly intervals throughout the timescale of the project and will report progress by way of minutes to FSA and will consist of RJ, SE, LV, JK, LV & GW. This project will be part of VLA-Weybridge's TSE Programme and as such will be managed under the TSE Research & Development and Surveillance Programme Management Group chaired by Dr Danny Matthews who will also attend project management meetings where possible. The project will also be subjected to the VLA monthly milestone management system, reporting to the VLA R&D Steering Group and the VLA Senior Management Group.

C7. Exploitation and Dissemination Plans

Dissemination of data and results on the presence (or absence) of abnormal PrP in the milk of cattle exposed to the BSE agent will be communicated to, and through, FSA in the first instance as quickly as possible commensurate with the needs for rigorous quality assessment and confirmation of those results. Subsequent reporting through peer reviewed journals will be essential

PART D – PROJECT FINANCES

((Details declared here will go to form ‘Section 4 - Pricing’ of the research contract if the proposal is successful))

The project lead-contractor should complete form FA1 (Proposal Cost Summary) which collates all participants costs and summarises them in a single table.

Each member of the project consortium (including the project lead contractor) is required to complete form FA2 (Participant Cost Summary) which details their individual costs.

The project lead contractor should collate all financial details into a proposal Annex.

These forms should remain anonymous and Participant No. should be used to identify costings.

FA1 – PROPOSAL COST SUMMARY

Participant No.	Project Year (not financial year)				
	Year 1 (£)	Year 2 (£)	Year 3 (£)	Year 4 (£)	Year 5 (£)
1					
2					
TOTAL YEARLY COST (£)					
VAT					

FA2 – PARTICIPANT COST SUMMARY (1 FORM PER PARTICIPANT)

Participant No. 01

	Project Year (not financial year)				
	Year 1 (£)	Year 2 (£)	Year 3 (£)	Year 4 (£)	Year 5 (£)
Pay Costs					
Consumables					
Equipment					
Travel Expenses					
Overheads (specify)					
Sub-contracts and consultancy					
Other costs					
Capital Charge					
Animals					
Inflation @ 2.5%					
TOTAL PARTICIPANT COSTS (£)					

VAT

Are you registered for VAT? (YES or NO)

If YES, what is your registration number.

The VLA has the same VAT registration number as core DEFRA and, as such, services provided within this combined entity are not subject to VAT.

FA2 - PARTICIPANT COST SUMMARY (1 FORM PER PARTICIPANT)

Participant No. 02

	Project Year (not financial year)				
	Year 1 (£)	Year 2 (£)	Year 3 (£)	Year 4 (£)	Year 5 (£)
Pay Costs					
Consumables					
Equipment					
Travel Expenses					
Overheads (specify) 75%					
Sub-contracts and consultancy					
Leics Univ					
Other costs					
TOTAL PARTICIPANT COSTS (£)					

VAT

Are you registered for VAT? (YES or NO)

If YES, what is your registration number.

PART E – DECLARATION

Declaration

I confirm that I have read this application and the Agency's standard contractual terms and conditions and that:

- (a) the Agency may show this application to third parties for the purposes of obtaining expert opinion on its scientific merits; and
- (b) if granted, the work will be accommodated and administered in our Organisation in accordance with the Agency's contractual arrangements. The staff gradings and salaries quoted are correct and in accordance with the normal practice of this Organisation.

IF ANY PART(S) OF THE STANDARD AGENCY TERMS AND CONDITIONS IS / ARE UNACCEPTABLE THEN THIS SHOULD BE DECLARED, IN WRITING, AS AN ANNEX TO THIS PROPOSAL.

(1) Head of Department

Signature Date

Name and initials

Organisation

(2) Administrative Authority

Signature Date

Name and initials

Position

Organisation

Full postal address

Name of project leader

Full postal address of project leader

Note: This application should be submitted by/through:

- (a) **the Head of Department;** and
- (b) **the officer who will be responsible for administering any funds that may be awarded.**

Each should sign the above declaration.

PART E – DECLARATION

Declaration

I confirm that I have read this application and the Agency's standard contractual terms and conditions and that:

- (a) the Agency may show this application to third parties for the purposes of obtaining expert opinion on its scientific merits; and
- (b) if granted, the work will be accommodated and administered in our Organisation in accordance with the Agency's contractual arrangements. The staff gradings and salaries quoted are correct and in accordance with the normal practice of this Organisation.

IF ANY PART(S) OF THE STANDARD AGENCY TERMS AND CONDITIONS IS / ARE UNACCEPTABLE THEN THIS SHOULD BE DECLARED, IN WRITING, AS AN ANNEX TO THIS PROPOSAL.

(1) Head of Department

Signature Date

Name and initials

Organisation

(2) Administrative Authority

Signature Date

Name and initials

Position

Organisation

Full postal address
Telephone No. (including STD code) Postcode
Ext.

Name of project leader

Full postal address of project leader
Telephone No. (including STD code) Postcode
Fax No. (including STD code) Ext.

Note: This application should be submitted by/through:

- (a) **the Head of Department;** and
 - (b) **the officer who will be responsible for administering any funds that may be awarded.**
- Each should sign the above declaration.

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PP1 - PARTICIPANT PROFILE / INFORMATION (1 FORM PER PARTICIPANT)

Organisation Details

Organisation	Veterinary Laboratories Agency – Weybridge		
Department	TSE Molecular Biology		
Address	Woodham Lane, New Haw, Addlestone, Surrey. KT15 3NB		
Telephone Number	01932 341111	Fax Number	01932 347046
Participant Role	Lead Contractor	Participant No.	1
Short Name	VLA		

Authorised Person

Title (Mr, Mrs, Ms, Dr, Prof.)	Mr		
Family Name	Jackman		
First Name	Roy		
Telephone Number	01932 357533	Fax Number	01932 357529
Email	r.jackman@vla.defra.gsi.gov.uk		

Project Staffing

Please list the names and grades of staff who will work on the project together with details of their specialism (including details of their 5 most recent relevant papers published).

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Roy Jackman Band B., Lead Contractor. Head: Immunochemistry Group, VLA-Weybridge.

Jackman R & Everest S J. 1997. Detection of CNS disease. Patent 2291968

Green AJE Jackman R Marshall TAV & Thompson EJ 1999. Increased S-100b in the CSF of some cattle with BSE. Veterinary Record. **145** 107 – 109

Silverlight JS Brown AJ & Jackman R 1999. Antisera to Tilmicosin for use in ELISA and for Immunohistochemistry. Journal of Agricultural Immunology **11** 321 – 328

Ansfield M Reaney SD & Jackman R 2000. Production of a sensitive immunoassay for detection of ruminant and porcine proteins, heated to >130oC at 2.7 bar, in compound animal feedstuffs (CAFS). J Agric Immunol. In Press: December 2000 issue

Ansfield M Reaney SD & Jackman R. 2000. Performance assessment and validation of a sensitive immunoassay for detection of ruminant and porcine heat stable proteins in compound animal feedstuffs. J Agric. Immunol. In Press: December 2000 issue.

Jackman R Everest SJ. 2000 Electrochemical detection of urinary metabolites in BSE. Journal of Chromatography Submitted

Jackman R and Everest SJ, 2000. Status of standard and novel diagnostic techniques for detecting TSEs. In: Proceedings of the 1999 Plenary Conference of the Animal Cell Technology Industrial Platform, 2000. pp 32 - 34

Sally J Everest, BSc, Band C. Group TSE Programme Manager, Immunologist and expert in the development and application of antibodies in analytical systems, Immunochemistry Group, VLA-Weybridge.

Leigh Thorne, MSc, Band D. Project leader and expert in PrP isolation and detection, Immunochemistry Group, VLA-Weybridge.

Anna Jenkins, BSc, Band E. Expert in DELFIA, CEA and Western Blot analysis of PrP^{sc}, Immunochemistry Group, VLA-Weybridge. Lindsay

Venables, HNC. Band E, Neuropathology Unit. (PA4). HNC Applied Biology. Manager: large scale TSE animal experiments.

PP1 - PARTICIPANT PROFILE / INFORMATION (1 FORM PER PARTICIPANT)

Organisation Details

Organisation	ADAS		
Department	Research Division		
Address	ADAS Rosemaund, Preston Wynne, Hereford HR1 3PG		
Telephone Number	01432 820444	Fax Number	01432 820121
Participant Role	Recombinant antibody development	Participant No.	02
Short Name			

Authorised Person

Title (Mr, Mrs, Ms, Dr, Prof.)	Mr		
Family Name	Kilpatrick		
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Project Staffing

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Please list the names and grades of staff who will work on the project together with details of their specialism (including details of their 5 most recent relevant papers published).

1. John Kilpatrick ADAS Biotechnology Segment Manager. Project Management
2. Dr Yi Li Senior Research Scientist. Molecular Biologist, located at University of Leicester

PUBLICATIONS

Li, Y., Cockburn, W. and Whitelam, G.C. (1998) Filamentous bacteriophage display of a bifunctional protein A::scFv fusion. *Mol. Biotech.* 9: 187-193

Li, Y., Cockburn, W., **Kilpatrick, J.** and Whitelam, G.C. (1999) Selection of rabbit single-chain Fv fragments against the herbicide atrazine using a new phage display system. *Food Agric. Immunol.* 11, 5-17

Gough, K.C., Li, Y., Vaughan, T.J., Williams, A.J., Cockburn, W. and Whitelam, G.C. (1999) Selection of phage antibodies to surface epitopes of *Phytophthora infestans*. *J. Immunol. Meth.* 228, 97-108

Li, Y., **Kilpatrick, J.** and Whitelam, G.C. (2000) Sheep monoclonal antibody fragments generated using a phage display system. *J. Immunol. Meth.* 236, 133-146

Li, Y., Cockburn, W., **Kilpatrick, J.B.** and Whitelam, G.C. (2000) High affinity scFvs from a single rabbit immunised with multiple haptens. *Biochem. Biophys. Res. Commun.* 268, 398-404

Li, Y., Cockburn, W., **Kilpatrick, J.** and Whitelam, G.C. (2000) Cytoplasmic expression of a soluble synthetic mammalian metallothionein-a domain in *Escherichia coli*. *Mol. Biotechnol.* 16: (in press)

Chapters

Gough, K.C., Li, Y. and Whitelam, G.C. (2001) Antibody phage display libraries. In 'Plant Molecular Biology: A Practical Approach'. Eds P. Gilmartin and C. Bowler. Oxford University Press. (in press)

PP1 - PARTICIPANT PROFILE / INFORMATION (1 FORM PER PARTICIPANT)

Organisation Details

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Short Name			

Authorised Person

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Project Staffing

Please list the names and grades of staff who will work on the project together with details of their specialism (including details of their 5 most recent relevant papers published).

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Prof Garry C Whitelam Head of Department, Molecular Biology

PUBLICATIONS

- Gough, K.C., Cockburn, W. and **Whitelam, G.C.** (1999) Selection of phage-display peptides that bind to cucumber mosaic virus coat protein. *J. Virol. Meth.* 79: 169-180
- Gough, K.C., Li, Y., Vaughan, T.J., Williams, A.J., Cockburn, W. and **Whitelam, G.C.** (1999) Selection of phage antibodies to surface epitopes of *Phytophthora infestans*. *J. Immunol. Meth.* 228, 97-108
- Hendy, S., Chen, Z.C., Barker, H., Santa Cruz, S., Chapman, S., Torrance, L., Cockburn, W. and **Whitelam, G.C.** (2000) Rapid production of single-chain Fv fragments in plants using a Potato Virus X episomal vector. *J. Immunol. Meth.* 231, 137-146
- Li, Y., Kilpatrick, J. and **Whitelam, G.C.** (2000) Sheep monoclonal antibody fragments generated using a phage display system. *J. Immunol. Meth.* 236, 133-146
- Li, Y., Cockburn, W., Kilpatrick, J.B. and **Whitelam, G.C.** (2000) High affinity scFvs from a single rabbit immunised with multiple haptens. *Biochem. Biophys. Res. Commun.* 268, 398-404
- Li, Y., Cockburn, W., Kilpatrick, J. and **Whitelam, G.C.** (2000) Cytoplasmic expression of a soluble synthetic mammalian metallothionein-a domain in *Escherichia coli*. *Mol. Biotechnol.* 16: (in press)

Chapters

- Whitelam, G.C.** (2000) Vaccine production in plants. In 'Fighting Infection in the 21st Century' eds. P.W. Andrew, P. Oyston, G.L. Smith and D.E. Stewart-Tull, Blackwell Science, pp 133-142
- Gough, K.C., Li, Y. and **Whitelam, G.C.** (2001) Antibody phage display libraries. In 'Plant Molecular Biology: A Practical Approach'. Eds P. Gilmartin and C. Bowler. Oxford University Press. (in press)
- Gough, K.C. and **Whitelam, G.C.** (2001) Production of antibodies using transgenic plants. In 'Plant Genetic Engineering Vol. 2: Applications and Limitations. Eds. R.P. Singh and P.K. Jaiwal (in press)

REFERENCES

1. Donnelly CA, Ferguson NM, Ghani AC, Wilesmith JW, Anderson RM. 1997. Analysis of dam-calf pairs of BSE cases: confirmation of a maternal risk enhancement. *Proc. R Soc. Lond. B. Biol. Sci.* 246: 1388, 1647 - 1656.
 2. Curnow RN, Hau CM. 1996. The incidence of BSE in the progeny of affected sires and dams. *Vet. Rec.* 138: 17, 407 - 408.
 3. Ferguson NM, Donnelly CA, Woolhouse ME, Anderson RM. 1997. A genetic interpretation of heightened risk of BSE in offspring of affected dams. *Proc. R. Lond. B. Biol. Sci.* 264: 1387, 1445 - 1455.
 4. Wilesmith JW, Wells GA, Ryan JB, Gavier Widen D, Simmons MM. 1997. A cohort study to examine maternally-associated risk factors for BSE. *Vet. Rec.* 141: 10, 239 - 243.
 5. Ferguson NM, Donnelly CA, Woolhouse ME, Anderson RM. 1997. The epidemiology of BSE in cattle herds in Great Britain. II. Model construction and analysis of transmission dynamics. *Philos. R. Soc. Lond. B. Biol. Sci.* 352: 1355, 803 - 838
 6. Taylor DM, Ferguson CE, Bostock CJ, Dawson M. 1995. Absence of disease in mice receiving milk from cows with BSE. *Vet. Rec.* 136: 23, 592
 7. Bradley R. 1993. The research programme on transmissible spongiform encephalopathies in Britain with special reference to BSE. *Vet. Rec.* 142: 7, 155 - 159
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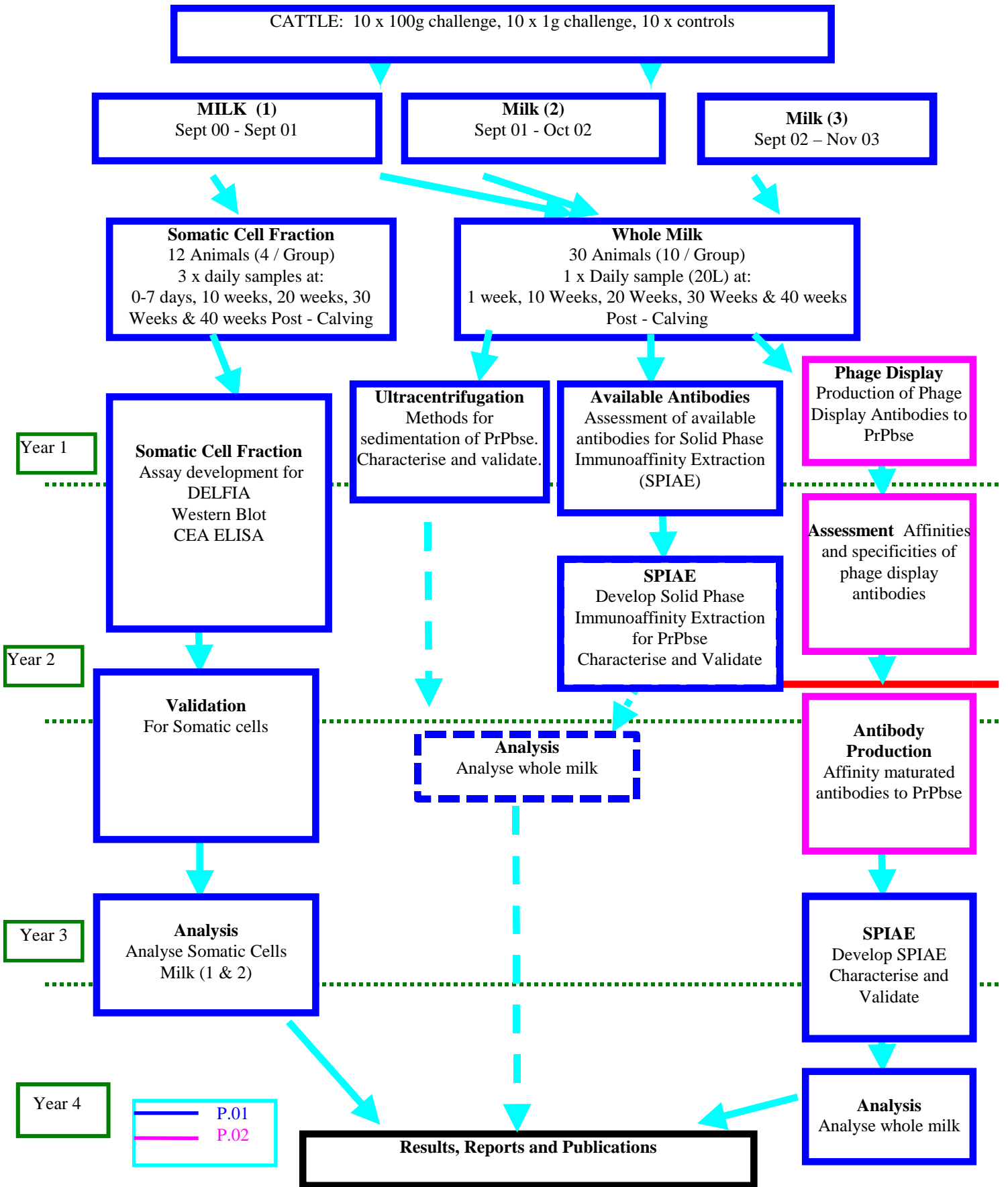


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FLOW CHART: MO3016, PrPbse in Milk



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APPENDIX B

SE 1736 Protocol Amendment

Number: 18
Section: 21/SE1736 – Milking and the collection of milk samples
Effective Date: 30 August 2000

Summary

A complete section has been added detailing how milking is to be carried out. This includes sampling and processing of colostrum and milk, identification and treatment of mastitis, maintenance of milking equipment, milk disposal, dry cow management and the recording requirement.

Reasons

To provide detail on how the heifers are to be managed during lactation.

Impact on study

This amendment will help ensure that the females on the study are managed according to the existing study guidelines, according to good agricultural and animal welfare practices while ensuring that the required samples are taken.

Signatures:

..... **Date:**.....

ADAS Study Director

..... **Date:**.....

VLA Sponsor Monitor

Distribution: Mr P N Johnson, ADAS Drayton
S A C Hawkins, VLA Weybridge



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21/SE1736

Title: Milking and the collection of milk samples.

Objective: To provide CVL with milk samples as required.

Field of application: ADAS Drayton.

Responsibility: ADAS Study Director.

Documentation required: Relevant recording forms and labels for sample containers.

1. Introduction

- 1.1 Females on the study will be managed in order to provide samples which will be collected according to a schedule compiled by the CVL Sponsor Monitor. To this end, female animals will be put in calf. It is the responsibility of the ADAS Study Director to oversee the management of the animals and to arrange both the artificial insemination together with the consequent husbandry and the collection of milk samples in accordance with the CVL schedule.
- 1.2 The equipment employed during milking and sample collection in each building is dedicated to that building and may not be removed, with the exception of the labelled sample containers and the recording forms.

2. Milking

- 2.1 In advance of calving, heifers should be walked through the milking parlour on a number of occasions. While in the parlour they should be fed a proportion of their daily concentrate ration and the milking machine should be turned on. This should help them to become familiar with the experience so that, when carried out in earnest, the milking procedure should be less traumatic.
- 2.2 Milking will be undertaken by trained, competent operators using a similar milking routine. Animals will be handled quietly at all times to minimise stress.
- 2.3 Milking must be conducted to good husbandry and hygiene standards. Control of mastitis is important to the study and a number of procedures have been incorporated into this protocol. If an animal shows any sign of illness or displays uncharacteristic behaviour, the possibility of mastitis should be considered.
- 2.4 In addition to regular protective clothing (overalls, wellingtons and disposable gloves), waterproof clothing, hats and safety spectacles will be provided.
- 2.5 Animals will be walked quietly to the milking parlour for milking, and returned to their pen by the same route.
- 2.6 Once in the milking facility area, each animal will be guided into one of the four standings where it will be confined by means of a chain fastened behind it. Each standing also has a head yoke as an additional means of restraint where necessary.
- 2.7 A proportion of the daily concentrate ration will be fed to each animal during milking.
- 2.8 Udders should be examined for swelling or inflammation of quarters, which may indicate mastitis.

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- 2.9 Once palpation of udders is complete, two to three streams of foremilk must be stripped into a strip cup from each teat, individually, and carefully examined for abnormalities such as clots or discoloration. Forestripping must always precede cleaning of udders.
- 2.10 Teats will be dry wiped with clean paper towel or, if slightly soiled, cleaned with disposable, medicated teat wipes. However, if badly soiled, teats and udders must be washed and thoroughly dried with paper towel.
- 2.11 The milk flow will be carefully monitored to ensure that the animal is neither under- nor over-milked.
- 2.12 The cluster may fall, or be kicked off before milking has finished. If dirty, it must be washed before being replaced for milking to continue.
- 2.13 Once milking is complete, the cluster should be removed and within 30 seconds, a post-milking iodine based teat disinfectant should be applied ensuring that the whole surface of each teat is covered. The teat dip should be a ready to use preparation containing 5000 ppm iodine and a minimum 10% emollient.
- 2.14 Following the milking procedure, animals will be returned to their pens. Hay should be freely available from the feed troughs, in an attempt to get them to stand for at least half an hour after milking whilst the teat orifice closes.

3. Colostrum Sampling

- 3.1 A sample of 100 ml of colostrum should be collected from each animal at each of the first four milkings after calving.
- 3.2 Colostrum can be stripped by hand directly into the decontaminated collection container or collected from the recording jar into the appropriately labelled sample container.
- 3.3 If the sample is to be collected from the recording jar, only the first animal to use that station at a milking can be sampled. Alternatively, the milk line and recording jar should be washed prior to sampling.
- 3.4 All samples need to be processed but they can be refrigerated for up to 24 hours prior to this.
- 3.5 When transferring samples across the step over bench in each changing room, the sample container should be wiped in a solution of 20% chlorox and placed in a clean plastic bag.
- 3.6 A combination of ADAS and VLA staff will be responsible for processing colostrum samples in the laboratory. When working with these samples a laboratory coat, disposable gloves and safety spectacles should be worn. In addition, a disposable bench guard should be placed on the work surface.
- 3.7 Each sample, including its container, should be weighed. An empty container and its lid should be placed on the balance and made up to the same weight with water.
- 3.8 The colostrum sample should be placed in the centrifuge and the water counter balance placed opposite. It should be spun at 3000 rpm for 10 minutes.
- 3.9 Using a pipette, the cream should be removed and placed in appropriately labelled universal tubes.

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- 3.10 The skimmed milk should then be removed using a different pipette and transferred to appropriately labelled universal tubes.
- 3.11 Any visible solids should be collected into a labelled universal tube.
- 3.12 The universal tubes should be placed in a -80°C freezer.
- 3.13 The soiled colostrum container should be soaked in a 20% chlorox solution for an hour, washed and then autoclaved.
- 3.14 All soiled pipettes, excess milk and the bench guard should be double bagged and disposed of as clinical waste.
- 3.15 In the three days following calving, one large sample of colostrum, 20 l maximum, should be taken. The CVL Sponsor Monitor should be consulted on the timing of this sample so that transport to CVL can be arranged. Sample containers should be labelled with the study ID, the animal ID, the sample type and date.
- 3.16 The sample should be collected directly from the recording jar and transferred to two 10 l containers. Samples should only be taken through cleaned milking stations. This can be achieved by taking it from the first animal to use a station at a milking or by washing the milking line before sampling. The latter method will be required in B2 and B3 when some animals have already gone through the three-day post-cleansing period.
- 3.17 If a heifer is likely to yield less than 10 l in a morning milking, the previous evening's yield should be collected in one 10 l container and the morning's yield in a separate 10 l container. On no account should the two samples be mixed.
- 3.18 Samples can be refrigerated in the changing rooms or in the A building laboratory to await collection. On taking the samples over the step over bench in the changing rooms, the 10 l containers should be placed in plastic bags.
- 3.19 Alternative refrigerated storage is available at ADAS NSRU, if required. To help prevent contamination of this refrigerator, samples should be placed in empty boot dip containers.
- 3.20 Details of all colostrum samples must be entered onto the Colostrum Sample Record.

4. Small Volume Milk Samples

- 4.1 Milk sample containers will be prepared prior to sampling. Each container should be labelled with the study ID, the animal ID, the sample type and date.
- 4.2 Three samples of 100 ml should be collected from each animal each month.
- 4.3 At any one milking occasion, only the first animal to use a particular standing should be sampled.
- 4.4 Milk samples should be transferred directly from the milk recording jars to the appropriately labelled sample containers.
- 4.5 The samples should be processed by VLA staff according to the procedure described at 3 for small volume colostrum samples.
- 4.6 Details of the samples collected must be entered onto the Small Milk Sample Record.
- 4.7 Additional small volume samples will be collected at times of mastitis and processed as above.

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5. Large Volume Samples

- 5.1 Milk sample containers will be prepared prior to sampling. Each container will be labelled with the study ID, the animal ID, the sample type and date.
- 5.2 Samples of up to 20 l will be collected from each animal at 3, 13, 26 and 39 weeks into lactation for homogenisation and pasteurisation (HP) by VLA staff. These samples should be as close to 20 litres as possible, the yield from two consecutive milkings can be mixed to achieve this.
- 5.3 Three samples of up to 10 l will be collected from each animal at 10, 20 and 30 weeks into lactation. The three samples should be taken within a week and all samples should be collected by VLA within 24 hours for “cells” processing at Weybridge.
- 5.4 On any one milking occasion, only the first animal to use a particular standing will be sampled.
- 5.5 The sample should be collected directly from the recording jar and transferred to 10 l container(s).
- 5.6 The CVL Sponsor Monitor should be consulted on the timing of sampling so that transport to CVL or processing at Drayton can be arranged.

6. Identification and Treatment of Mastitis

- 6.1 Mastitis is inflammation of the udder, commonly caused by infection, stress and physical damage. The possibility of mastitis must be considered when an animal is “off-colour,” has an elevated temperature or is behaving in an uncharacteristic manner. It is generally individual quarters that initially become infected and not whole udders. Mastitis can be categorised into sub-clinical and clinical.
- 6.2 In a sub-clinically infected quarter the milk may have an elevated somatic cell count (SCC), but the milk and udder tissue will appear normal. It is likely that mastitis pathogens can be isolated from the milk.
- 6.3 In a clinically infected quarter the SCC will be elevated, udder tissue may appear swollen and the milk will appear abnormal. It is likely that high levels of mastitis pathogens can be isolated from the milk. The animal may show signs of disease including lethargy and elevated temperature.
- 6.4 A milk sample will be taken routinely each month from each animal for SCC in an attempt to identify trends and any sub-clinical mastitis. Any animal with a SCC above 400,000 will undergo a more comprehensive examination of udder health in which two samples will be taken from each quarter. A California Milk Test will be conducted on one of these and the other will be submitted to VLA for bacteriological culture and sensitivity.
- 6.5 Animals with a SCC above 400,000 should, if possible, be milked last. Alternatively, the cluster should be disinfected in a 1:200 solution of peracetic acid before milking the next cow to help prevent cross infection. Cows with consistently high SCC should be considered for early drying off.

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- 6.6 Where mastitis is suspected, two samples from the infected quarter will be collected immediately. A California Milk Test will be conducted on one of these and the other will be submitted to VLA for bacteriological culture and sensitivity.
- 6.7 During milking care must be taken not to spread infection through equipment, operator handling or splashing of foremilk or water. Teat preparation must be carefully undertaken. Gloves must be changed immediately after contact with an infected quarter or animal. Any equipment potentially contaminated by an infected quarter must be thoroughly disinfected immediately afterwards. Clusters used to milk a mastitic animal must be completely immersed in a 1:200 solution of peracetic acid.
- 6.8 Animals with mastitis should be housed in isolation and milked last where possible, to minimise the possible spread of infection to others.
- 6.9 Where a case of mastitis is suspected the NVS will be called immediately for diagnosis and prescription of treatment. Cases of clinical mastitis will be treated without delay for at least seven days and for at least 72 hours beyond a clinical cure.
- 6.10 Where the NVS recommends intramammary infusion of teats, the operator must be experienced or supervised by another experienced operator. The animal should be restrained firmly with special regard to both the safety of the operator and the health and welfare of the animal. Animals should be milked before infusion (unless dry). Teats must be clean and dry and, if there is infection in more than one quarter, separate syringes must be used for each. Therefore, after milking, teat and teat orifices to be infused should be carefully disinfected with a cotton wool swab soaked in surgical spirit or a disposable, medicated wipe, starting with the furthest pair of teats and moving onto the nearest pair. The protective cap should be removed from the syringe exposing the cannula. Infusion should take place in reverse order to pre-disinfection, so the cannula should be introduced into the most recently cleaned teat via the sphincter and the contents of the syringe expelled by depression of the plunger. The infused quarter must then be gently massaged and the teat dipped in an iodine-based disinfectant which should then be discarded. The infusion procedure should be repeated for all infected quarters.
- 6.11 A case of mastitis is defined as one quarter infected once. Therefore, an animal with two quarters affected at the same time counts as two cases. If a quarter relapses following treatment, within 7 days of the end of treatment, this is classed as a continuous case. However if mastitis recurs in the same quarter after the 7-day treatment period, it is classed as a new case.
- 6.12 Full details of mastitis incidence and treatment will be recorded in the Study Diary Record and Veterinary Medicine Administration Record forms. These records will be reviewed at six monthly intervals in conjunction with SCC records in order that trends and problems can be addressed. Cows that have three or more cases of mastitis in one quarter or more than five cases in total in a lactation should be considered for culling.

7. Dry cow management and therapy

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- 7.1 The environment of the dry cows will be managed to ensure that teats are kept clean.
 - 7.2 Fly repellent will be administered routinely.
 - 7.3 Drying off should be an abrupt process when the animal is being milked daily. Yield will be quickly suppressed at drying off by limiting diet for 24 hours and, where possible, removal of the animal from the milking herd, to minimise the milk production stimulus. Dates of commencement of lactation and drying off will be recorded on the Study Diary Record.
 - 7.4 Drying off should take place approximately two months before the next calving.
 - 7.5 All animals will receive dry cow therapy at the end of a lactation. Udders will be infused immediately after the last milking, which will have flushed the teat canal, using the procedure outlined at 6.10.
 - 7.6 The udders of dry cows will be examined by palpation on a monthly basis. However, it is important not to strip out quarters unless mastitis is present, in order to avoid breaking the seal which develops at the teat orifice during the dry period. Visual checks will be made daily.
 - 7.7 The teats of animals will be dipped for the last 7 – 14 days of any dry period.
 - 7.8 Bacterial sensitivities will dictate the choice of dry therapy after the first lactation.
- 8. Records to be kept during milking and milk sampling**
- 8.1 Changes in the diet of each female during pregnancy and lactation will be documented on the Feeding Record. This will incorporate details of dates of ration introduction, concentrate mix identity and amount offered.
 - 8.2 The Study Diary Record and Veterinary Administration Record should, between them, contain full details of mastitis incidence and treatment i.e. cow identity, date, quarter(s) infected and treatment applied.
 - 8.3 Milk yield for each cow will be recorded on the Milk Yield Record at each milking.
 - 8.4 Details of all milk samples collected will be entered onto the Colostrum Sample Record and the Milk Sample Record.
 - 8.5 Details of the results of Somatic Cell Counts (SCC) will be entered onto the Access database for easy access and analysis.
- 9. Cleaning and maintenance of the milking equipment and parlour**
- 9.1 After each milking occasion the cow standings and milking equipment will be thoroughly cleaned. Some of the chemicals used are hazardous. The appropriate personal protective equipment must be worn at all times during handling of these potentially hazardous substances. Copies of the safety data sheets for all hazardous chemicals will be kept in the C buildings.
 - 9.2 The cow standings and collection/handling areas will be thoroughly hosed down.
 - 9.3 The external surfaces of the clusters and associated pipe work will be thoroughly washed clean.

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- 9.4 The clusters should then be placed on the jet sets, the delivery pipe directed onto the floor and the system will be flushed with cold water, drawn from the wash tank, to remove residual milk traces, with the water running to waste.
- 9.5 Following the cold rinse, water heated to at least 85°C, will be used to fill the wash tank. This hot water will then be drawn into the system until the water discharging to waste is hot 50°C.
- 9.6 At this point, the end of the delivery pipe is connected to the circulation system a detergent chemical e.g. Circlean WS is added to the wash tank. The solution will then be circulated throughout the system for a minimum of 7 minutes.
- 9.7 Once this time has elapsed, the delivery pipe will be disconnected from the circulation system and re-directed onto the floor. The hot solution in the system will then be emptied to waste.
- 9.8 The cleaning routine is completed with the delivery pipe being once more connected to the circulation system whilst the wash tank is filled with cold water to which hypochlorite is added at a rate of 25ml in 40 litres. This is then circulated throughout the system and then discharged onto the floor.
- 9.9 At every 7th wash, a chemical incorporating a milk stone remover e.g. Westfalia Single Action will be used in the circulation cleaning routine instead of the detergent chemical.
- 9.10 The milking installation should be serviced by an adequately trained operator, in accordance with the equipment service schedule prepared by the manufacturer i.e. Westfalia Regal Care.
- 9.11 An assessment of the performance of the system should be undertaken at 6 monthly intervals and daily checks should be made of the vacuum level, air bleeds and pulsator operation. These tasks will be carried out by ADAS.
- 9.12 Teat liners should be checked daily and changed a minimum of every six months.
- 9.13 A supply of parts likely to require regular replacement will be maintained at ADAS Drayton.

10. Milk Disposal

- 10.1 Milk should be discharged from each milking parlour to the 1000 l bulk storage tank located within each isolated unit. These tanks can be moved using the fork lift attachments for the JCB.
- 10.2 In B1, milk can be discharged directly to the underground tank without treatment. Whereas in B2 and B3, the milk should be treated with chlorox at a rate of one part chlorox to four parts milk, left an hour and then discharged to the underground tank.
- 10.3 In all cases the milk tank should be moved so that it is directly above the underground tank before discharge.

SE 1736 Small Milk Sample Record

Building B

Animal Number :

	Date	am/pm	Sample 1	Sample 2	Sample 3	Signature
4 weeks						
9 weeks						
13 weeks						
17 weeks						
22 weeks						
26 weeks						
30 weeks						
35 weeks						
39 weeks						
43 weeks						

SE 1736 Large Volume (Homogenisation / Pasteurisation) Sample Record

Building B

Animal Number :

	Date	am/pm/both	Approximate quantity (l)	Signature
3 weeks				
13 weeks				
26 weeks				
39 weeks				

SE 1736 Large Volume (Cell Fractionation) Sample Record**Building B****Animal Number :**

	Date	am/pm/both	Approximate quantity (l)	Signature
10.1 weeks				
10.2 weeks				
10.3 weeks				
20.1 weeks				
20.2 weeks				
20.3 weeks				
30.1 weeks				
30.2 weeks				
30.3 weeks				

APPENDIX C

Processing and archiving of milk from cattle experimentally challenged with BSE.

1. Introduction.

Milk and colostrum is collected from live cattle to provide samples for future tests, see ADAS milking protocol. The milk may be processed to separate the cream, milk, and cell fraction prior to freezing, and milk may also be homogenised and pasteurised prior to freezing.

2. Safety.

2.1. "Guidelines for Working with TSE's in Pathology Laboratories" should be referred to before any procedure is carried out. Milk as a body fluid is covered by Neuropath risk assessment 25. All relevant protective clothing must be worn whilst preparing these samples, i.e. laboratory coat, gloves and safety glasses.

2.2. Working with large animals can be dangerous if they are not handled and restrained correctly. ADAS staff regularly milk cattle following ADAS protocols.

3. Materials.

Freezer proof collection bottles, containers, and labels.

-80 freezer.

Protective clothing; laboratory coat, gloves and safety glasses.

Centrifuge for spinning samples at 3000 RPM for 10 minutes.

Pastettes – 5ml

Homogeniser.

Pasteuriser.

4. Procedure.

Animals are milked regularly by ADAS staff. At pre-arranged sampling times milk from identified animals is collected into suitable containers, directly from the parlour into labelled bottles. These samples may be refrigerated for up to one day, before either processing or freezing direct. Only the first animal to use each milking parlour station may be sampled, and the milking parlour is cleaned after each milking session. Small samples of colostrum may be taken by hand. Animals which have cleansed must be sampled before those which have yet to cleanse.

Animals that are large volume milk sampled, will be blood sampled (1 x clotted, 2 x EDTA, 1 x oxalate fluoride) within one week of the sampling date. Blood samples can be stored in a fridge for one day before transport to Roy Jackman.

Colostrum and whole milk collection. – small volume.

100 ml of colostrum will be sampled at 12, 24, 36 and 48 hours after birth. This will be spun at 3000 RPM for 10 minutes to separate the cream, and the cream, milk and any visible solids frozen separately in labelled universals. The colostrum sampled at 36 hrs will be labelled and frozen separately for Mark Rogers.

3 x 100 ml milk will be sampled monthly, and processed as above before freezing. 100 ml of this monthly collection will be frozen separately for Mark Rogers.

Extra 100ml samples will be taken from animals with mastitis. These will be hand stripped from the infected quarters prior to milking on the first three consecutive days. These extra samples will be processed as above.

Homogenised and Pasteurised milk collection. – large volume HP

Large volume milk samples (20 l) will be collected at 2 to 3 weeks after calving, and at 3, 6 and 9 months through to the end of lactation. The 20-l samples will be collected from a mixture of morning or afternoon milkings, and a smaller volume collected where 20 l is not available. The milk will be passed through a Homogeniser and Pasteuriser, before being frozen at -80.

Operation of the PASTEURISER and HOMOGENISER units.

Turn both units on at wall.

On pasteuriser turn mains switch to ON.

HALF FILL PASTEURISER HOPPER WITH WATER.

Open cooling valve on pasteuriser side.

Pasteuriser - set heater to 92, and check boiler is set at 96.

Connect outflow from Homogeniser (clear tube) to pasteuriser hopper.

Check pasteuriser outflow (metal tube) is connected to Homogeniser inflow.

Check there is water in the pasteuriser hopper.

Turn Homogeniser ON (on top).

Press on/zero button on Homogeniser pressure gauge.

Set Homogeniser pressure to 40-50 by adjusting pressure adjustment valve. Do not touch the top valve.

Turn pasteuriser pump ON.

Turn pasteuriser heater ON.

Throughout operation of the pasteuriser, if red pump light comes on warning of low pump water levels, then add water by opening side valve till excess runs out of the side drain.

When heating temperature reaches 92, remove clear pipe from hopper and lead into waste container.

As water empties from the pasteuriser hopper, add some milk sample.

THE HOPPER MUST NEVER BE ALLOWED TO EMPTY.

When milk flows from the waste pipe, start sample collection into a freezer-proof container.

Fill hopper to brim with milk, and set alarm to 15 minutes.

The alarm will warn of the hopper emptying. Keep replenishing the sample hopper till sample is complete. When the processed sample container is full (leaving expansion space), replace with another container.

As the last of the sample exits the hopper, run in water, a little at first, then half fill the hopper with water.

Finish sample collection as the water is run in, and remove waste pipe to waste container.

Water is run through the pasteuriser and Homogeniser between individual samples.

As the water leaves the hopper, run in the next sample, slowly at first.

Do not start collection of the next sample until pure milk is flowing from the pipe.

Several samples may be processed in one day, but always in order – group 1 then group 3, and group 2 last.

At the end of the sampling day, 2% H Na₂ O can be run through as a product run to clean the equipment. To do this set the heating temperature to 80, turn off the cooling water, and run the Homogeniser outflow (clear tube) into the pasteuriser hopper. Reduce Homogeniser pressure to zero.

Allow 20 minutes for this cleaning run, then move the Homogeniser outflow to lead into the sink, and as the 2% H Na₂ O leaves the hopper, run through with a hopper of water.

If no cleaning in place is required, then turn off the Homogeniser, then the pasteuriser heater, then pump then turn off the pasteuriser. Turn both off at the wall.

CIP. Clean in place.

The pasteuriser can be further cleaned in place, but not the Homogeniser.

Disconnect the metal tube from the Homogeniser, and lead it into the pasteuriser hopper. Turn off Homogeniser.

Fill pasteuriser hopper half full of cleaning fluid.

Turn pasteuriser key from process to CIP, and run for 20 minutes.

After this cleaning run, lead the metal tube from the pasteuriser hopper to the sink, ensuring no spillages. As cleaning fluid leaves the hopper, fill the hopper with water.

Turn off the pasteuriser heater, and when water level is low in the hopper, turn off the pump.

Return key from CIP to Process, ready for future work.

To drain excess water from pasteuriser hopper, ensure pump is off, then turn side lever from Process to Drain, and collect the waste water from side pipe.

Collection for cell fraction. – large volume cells.

Large volume samples will be collected within the first three days after calving, and at peak lactation (10 weeks), and at 20 and 30 weeks after calving. Four animals will be sampled through lactation, on dates decided by CVL. These samples will be transported to CVL (Roy Jackman) within 24 hours of sampling, for fractionation.

All samples will be taken direct from the milking parlour.

All samples will be transported in 10 Lt containers, with tap secured by tape, and container enclosed in a polythene bag.

5. Contingencies.

If sample collection is arranged for mid-day then the daily sample may be a combination of that mornings and the previous evenings milkings.

If the required volume of milk cannot be collected, collect what is available for the sample.

Sampling stops at end of lactation, and repeats following further calvings.

Sampling requests may change throughout the project life.

Sampling to be delayed in cases of cattle illness.

(see "Heifer management protocol")

APPENDIX D

Meeting to discuss research on the “Determination of abnormal prion protein in the milk of cattle exposed to the BSE agent”.

2:00pm, 7 August 2000 in Room 326, Ergon House, Horseferry Road, London.

Present: Steve Dixon (Chairman), Petre Kwasowski, John Kilpatrick, Mary Howell, Donald Muir, Mike Dawson, Lindsay Venables, Roy Jackman.

Background and purpose of the meeting: In April 1998 SEAC identified this as an area where further information was needed. This was taken forward at the November 1998 meeting of SEAC and subsequently advertised as a MAFF research requirement for funding in 2000/01. The MAFF appraisal of the proposal raised several issues that were largely supported by the FSA to whom the responsibility for commissioning this research was transferred on April 1, 2000.

The purpose of the meeting was to consider the research proposal submitted by the VLA (Determination of abnormal prion protein in the milk of cattle exposed to the BSE agent) with the object of developing a more focused project and clarification of the scientific approaches

Main points:

- ◆ It is important that the study should provide robust scientific data that would prove or disprove the presence of the abnormal prion in the milk of cattle exposed to the BSE agent.
- ◆ The main issues of concern to the FSA regarding this proposal are:
 - Milk sampling/storage and processing prior to analysis
 - Antibody production
 - End point detection systems
 - Data quality
- ◆ Current methods of analysis are validated for diagnosis of BSE in cattle brain. These are immunoassays that utilise antibodies that recognise only the denatured PrP (BSE). No antibodies have been produced that are able to recognise the native PrP (BSE).
- ◆ No assay exists for the analysis of PrP (BSE) in milk and therefore milk samples will need to be stored in some form until sample treatment protocols and endpoint detection systems are developed, validated and are able to provide reliable results.
- ◆ The chemistry and composition of milk is complex and changes throughout the lactation period. This is compounded by the fact that the mammary gland is particularly “leaky” during very early (2/3days post calving) and late lactation when milk will contain plasma components (proteinases, somatic cells etc.).
- ◆ A major objective for the study is the development of a pre-analysis sample processing protocol. Ideally the daily fresh milk samples should be fractionated and analysed for the presence of PrP (BSE). However it was always very unlikely that the timing of this project would allow this and as such the milk will be stored prior to analysis. A range of options was considered (freezing whole milk, lyophilisation, partial fractionation - continuous centrifugation, chemical treatment and homogenisation/pasteurisation). It was agreed that given the volumes of milk to be handled and the need to avoid “churning”, the best option would be homogenisation/pasteurisation followed by freezing. However freezing will cause changes that include cell lysis and so the option to separate cell fractions and other components by centrifugation after freezing will not be comparable with fresh milk. In particular, the separation of milk into cream and skim components will not be possible after homogenisation and may lead to fat-associated matrix interference in the endpoint analyses.
- ◆ Subsequent processing (fractionation) of the milk samples will consider physical and chemical treatments. These separations will be necessary to enable presentation of a purified milk extract to the end point detection system, but the ability to attribute activity to any fraction of fresh milk will not be possible.
- ◆ There is strong evidence that PrP is expressed by lymphocytes and that abnormal PrP is associated with the buffy coat fraction of whole blood in some TSEs. It was agreed that milk samples collected at time zero (day 1), 10 weeks and 30 weeks post calving, would be centrifuged to isolate somatic cells. These would be analysed for the presence PrP (BSE) utilising existing fractionation protocols, PrP denaturing conditions and immunoassays. There may be a delay in reporting these results if problems are encountered with the method for the release of any PrP bound to this

fraction. It is anticipated that preliminary results may be available within 4 / 5 months from the start of the project. The centrifugation supernatant will be homogenised, pasteurised and stored frozen to awaiting further fractionation and analysis.

- ◆ The method developed for whole milk is likely to be based on immunoaffinity extraction (IAE). However, it may be necessary to develop procedures that remove the vast majority of contaminating proteins without reducing the concentration of PrP, before more targeted extraction methods such as IAE are employed. The IAE options are either batch processing or columns, however both will rely on the availability of appropriate quantities of high affinity antibodies. The supply of suitable antibodies is a problem as none currently exist to the native PrP (BSE). Denaturing the fresh milk fractions with strong chaotropic reagents (urea, guanidine) to release the denatured prion, prior to IAE treatment is not a realistic option as other milk proteins will also be denatured and interfere with the isolation of PrP. There is a strong case for the development of antibodies to native PrP (BSE).
- ◆ Antibody production. Antibodies currently available from all sources have been generated to the denatured PrP (BSE) or small peptides that replicate a variety of epitopes including the main hydrophobic sites on the PrP molecule. These antibodies form the basis of all diagnostic tests developed to date and require a denaturing step prior to immunoassay. There are two issues to be considered a) the availability and need to generate more antibodies (not to different antigens) for the endpoint detection systems, and b) to generate antibodies to the native PrP (BSE) for IAE sample preparation. The former is unnecessary as there are sufficient antibodies available. However a limited evaluation of these antibodies may be need to be undertaken with the object of selecting the most suitable antibody for the analysis of milk extracts. The latter is a more formidable task. It was agreed that for native PrP (BSE) there was within this project, scope for the development of polyclonal, monoclonal and recombinant antibodies. It was noted that although the development of recombinant antibodies was very high risk, it was an option that might provide a solution given the failure of more traditional antibody preparation techniques. It was agreed that this facet of the programme was the most challenging and would need to be monitored carefully. If successful there would be far reaching implications particularly with regard to the IP.
- ◆ Regarding the validity of the data generated by the study, it is imperative that the work is conducted to the highest level of competence (GLP). In particular the analytical methods employed must be validated to internationally accepted standards (ISO, OIE). The FSA Data Quality Unit has agreed to provide advice and guidance on this.
- ◆ The importance of this project to the FSA is such that it will be monitored closely, its progress assessed and if necessary elements within the research programme terminated or the emphasis changed as results emerge. A review of the direction of the project may be necessary following the reporting of the results of the somatic cell study (above).

Action points:

1. Liaison between **Prof Muir and Roy Jackman** regarding the fractionation of milk samples.
2. Generation of antibodies – polyclonal/monoclonal **Roy Jackman/ Petre Kwasowski**.
3. Collection and storage of milk samples – **Lindsay Venables**
4. Immunoaffinity separations – **Roy Jackman/Petre Kwasowski**
5. Provision of advice on Data Quality (analytical) – **Mary Howell**
6. Revision of the research proposal – **Roy Jackman / Prof Muir / Petre Kwasowski / John Kilpatrick / Lindsay Venables**.

Steve Dixon 16/8/00

APPENDIX E

Ref:

To: Roy Jackman

From: Steve Dixon

Copies: Chris Lawson
David Taylor
Peter Hewson
+ file copy

Date: 16 August 2000
Room: 409b. LER
Phone: GTN: 238 6358

**MEAT HYGIENE (TSE) RESEARCH: DETERMINATION OF ABNORMAL PROION PROTEIN
IN THE MILK OF CATTLE INFECTED WITH THE BSE AGENT.**

1. I refer to your e-mail of the 15 August 2000.
2. I enclosed a note of the meeting to discuss the above research project and would appreciate any comments you might wish to make. It is unfortunate that the original research proposal did not contain sufficient baseline information to allow a full appreciate of all the issues. However during the meeting the reasons for the scientific approaches you were proposing became much clearer. The inclusion of Professor Muir (lactation and milk chemistry) and Dr Kwasowski (bio-process technology) will bring additional and valuable expertise to the project. The outcome of this is that the project will be more focused and I am confident that it now has a better chance of delivering usable results. The importance of this project can not be overstated and the results must provide clear and unequivocal conclusions.
3. Whilst there is a strong case for the development of more antibodies, this must not be an overriding priority for the study. This element of the study represents a significant resource commitment and will be monitored closely to ensure that it does not achieve its objectives at the expense of others.
4. Turning to the sampling of cattle at time zero (day1), 10 and 30 weeks post calving. I recollect that we agreed to only isolate the cellular fractions from a single milk sample from each animal at those time points. Although this will provide useful information, I would like you to open up the "sampling window" and isolate cellular fractions on each day over a seven-day period at each time point and also include an additional time point at 20 weeks. I have discussed the sampling protocol with Don Muir and he has suggested that the cellular fraction should also be isolated from any cows that develop mastitis. Despite the possibility that the subsequent analysis of those supernatants will be difficult, the data obtained on the cellular fractions will be more robust. I would be grateful if you could advise on the practicability and cost of the additional work.
5. The magnitude of the task and the problems that must be overcome are clear. It is therefore important that the project will need to be monitored carefully to ensure that the objectives are achieved within the time scale. The Agency would normally only expect annual and final reports, however I would like you to include, in the project milestones, 6 monthly progress update reports to the Agency together with an annual project review meeting – this should be scheduled to follow the submission of the annual/final report. The intention is that the annual review meeting will be a critical review of progress and would if necessary enable the Agency to change the emphasis or terminate elements of the work programme.
6. I am able to confirm that it is the intention of the FSA to proceed with the commissioning of this research project at the VLA. However as you have acknowledged, the final contract will be subject to the receipt and agreement on the revised research proposal you are preparing. It would be helpful if you could complete and return to me the FSA research application form RCUA3.doc (enclosed) both as a hard copy and on disc, as soon as possible.

Steve Dixon

**PROCESSING AND ARCHIVING OF MILK FROM CATTLE
EXPERIMENTALLY CHALLENGED WITH BSE**

Each challenge group has its own dedicated milking parlour to eliminate cross contamination between groups and only the first animal to use each milking parlour station is sampled during a milking session to eliminate the chances of cross contamination between animals in a given challenge group. The milking equipment is cleaned after each milking session with Circlean WS (sodium hydroxide/sodium hypochlorite) and every 2-3 days with Westphalia Single Action (phosphoric acid).

At pre-arranged sampling times 5 litres of milk will be collected from each of the animals on the study into pre-labelled polypropylene containers. This will provide 100g of cream and 1 litre of homogenised milk. The containers are then transferred to the laboratory facility. Samples from only one challenge group are processed at any one time.

1 litre of the milk is decanted into a polyethylene container. This is then covered and allowed to stand overnight at +4°C to allow the cream to rise. 100mls of cream are then removed by sterile pastette into a Nalgene and frozen at -80°C.

1 litre of milk is run through the homogeniser (working at 100 Bar), subaliquoted into 250ml Nalgenes and frozen at -80°C. The homogeniser is cleaned between samples by circulating a 2% NaOH solution through the system for 30 minutes followed by two clean water rinses.