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Development of a Cloth-Based Hybridization Array System for the Detection and Identification of Ruminant Species in Animal Feed

By

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A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Master of Science

College of Natural Sciences (CNS)

Department of Biology

Carleton University

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**Development of a Cloth-Based Hybridization Array System for the
Detection and Identification of Ruminant Species in Animal Feed**

Submitted by

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in partial fulfilment of the requirements
for the degree of Masters of Science

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ABSTRACT

A cloth-based hybridization array system (CHAS) for the rapid detection and identification of the principal ruminant species (bovine, sheep, goat, elk and deer) which may occur in feeds has been developed. In this approach, universal polymerase chain reaction (PCR) primers targeting conserved mitochondrial DNA sequences are used to amplify DNA extracted from feed samples, with subsequent detection and characterization of the PCR products by a low density array technique based on the use of polyester cloth as a solid support for the immobilization of oligonucleotide capture probes targeting species-specific variable sequences within the universal amplicons. Digoxigenin-labeled amplicons generated in the universal PCR were hybridized with an array of probes immobilized in discrete spots on a polyester cloth strip, followed by sequential reactions of the strip with anti-digoxigenin antibody-peroxidase conjugate and chromogenic substrate solution. The CHAS provided sensitive and specific detection of meat meal from the principal ruminant species blended in feeds, while enabling the identification of PCR-generated amplicons from the different species.

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LIST OF ABBREVIATIONS

BFM	blank feed matrix
BSA	bovine serum albumin
BSE	Bovine spongiform encephalopathy
°C	degrees Celsius
CFIA	Canadian Food Inspection Agency
CHAS	cloth-based hybridization array system
CJD	Creutzfeldt-Jakob Disease
cm	centimetre
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dUTP	deoxyuridine triphosphate
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay
fg	femtogram
GPI	glycoinositol phospholipid
M	molar
MBM	meat and bone meal
mg	milligram
min	minute
ml	millilitre

μ M	micro molar
mM	millimolar
NCBI	National Center for Biotechnology Information
ng	nanogram
PBS	phosphate buffered saline
PBST	PBS containing Tween 20
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
pg	picogram
POD	horseradish peroxidase
PrP	proteinaceous infective particle
PrP ^c	cellular prion protein
PrP ^{Sc}	scrapie prion protein
rpm	Revolutions per minute
Rxn	reaction
SSM	simulated meal meals
TMB	tetramethylbenzidine
Tris	hydroxymethylaminomethane
TSE	Transmissible spongiform encephalopathy
UV	Ultra violet light
vCJD	variant Creutzfeldt-Jakob Disease

Chapter 1

General Introduction

The year 1986 marked the emergence of a new food-related human health hazard that would become a major international political and economic issue. Bovine Spongiform Encephalopathy (BSE), a neurological disease affecting cattle first appeared in the United Kingdom, where a common source outbreak produced an epidemic (Nathanson et al, 1997). The emergence of this disease in cattle caused consumer panic that cost billions of dollars in lost revenues to the British beef industry, and led to stringent containment efforts to limit the spread of contamination.

BSE belongs to the family of Transmissible Spongiform Encephalopathy (TSE's) that are characterised by 'plaque' lesions and collections of proteinaceous material in the brain producing the characteristic 'spongiform' appearance upon microscopic examination. The disease manifests clinically with ataxia and dementia, and is incurable and fatal (Horwich and Weissman, 1997). It is the behavioural exhibition of ataxia and dementia that led to the coining of the phrase 'mad cow,' the name by which the disease is commonly referred.

The existence of TSEs affecting livestock and humans has been documented since the early 1800's, as is the case with Scrapie a TSE afflicting sheep. Human TSE's such as Creutzfeldt-Jakob Disease (CJD), Gerstmann-Strausler-Scheinker (GSS), and Fatal Familial Insomnia (FFI) have been documented since the 1920's. Even a human epidemic TSE form called Kuru was identified in 1957 among a cannibalistic tribe in New Guinea, although the incidence of Kuru among this tribe has all but disappeared upon cessation of cannibalistic practices.

For the most part human TSE's are rare and sporadic, and have been identified as a spontaneous human disease in which susceptibility might be inheritable. The onset of the disease is considered random and generally manifests itself in people after age 40 (Horwich and Weissman, 1997). There is a form referred to as iatrogenic form of CJD which results from transmission during a medical procedure. In spite of the occurrence of several different known TSE's inflicting humans the identification of a new strain potentially transmissible through consumption of infected beef caused serious public alarm.

Shortly after the appearance of BSE among cattle, variant Creutzfeldt-Jakob Disease (vCJD) was identified in humans. Following the BSE epidemic a CJD epidemiological surveillance program was re-instituted in the UK to identify changes in the occurrence of CJD cases (Will et al 1996). CJD cases in the UK had been surveyed since the 1970s and information such as the number of afflicted individuals and age at death had been collected. Upon renewed surveillance of the epidemiology of the disease, cases bearing a new neuropathological profile were identified, and named variant CJD (vCJD).

Electroencephalogram (EEG) changes normally seen in CJD-afflicted individuals were not present in patients suffering from vCJD. The afflicted individuals died at a much younger age, with the average age around 30yrs, as opposed to the average CJD age of death being 63yrs (Will et al, 1997). VCJD had much longer illness duration with

a mean of 13 months as oppose to 5 months for CJD, and the clinical findings were also different (Will et al, 1997). As opposed to the ataxia and dementia associated with CJD, individuals bearing vCJD presented psychiatric symptoms such as depression, anxiety and withdrawal, followed by ataxia and memory disturbances at much later stages of disease progression (Will et al, 1997). With respect to neuropathological symptoms, vCJD individuals showed “amyloid plaque formation extensively distributed throughout the cerebrum and cerebellum” (Will et al, 1997) whereas CJD showed 5-10% of individuals to possess amyloid plaques. (Amyloid meaning a hard waxy deposit consisting of protein and polysaccharides resulting from the degeneration of tissue).

At the time no other variant CJD cases had been reported outside the UK despite other countries having similar CJD monitoring programs. The lack of similar cases from other countries indicated the occurrence of vCJD to be potentially linked to conditions unique to the UK. The UK was the site of the highest BSE rate and it was suggested that humans had contracted vCJD by eating BSE tainted beef. BSE had crossed the species barrier and was now a threat to human health (Horwich and Weissman, 1997).

As of March 4 2005 the UK Department of Health reported 149 deaths from vCJD, as published in their monthly Creutzfeldt Jacob disease statistics. During the first year of surveillance in 1995 three cases were reported. The number of deaths peaked at twenty-eight in the year 2000. The number of deaths attributed to vCJD have since declined and only nine deaths were reported for 2004. The number of vCJD individuals currently alive is five (Creutzfeldt-Jakob Disease in the UK By Calendar Year, 2005

http://www.dh.gov.uk/PublicationsAndStatistics/PressReleases/PressReleasesNotices/fs/en?CONTENT_ID=4105346&chk=mCugjd).

Two theories have been proposed to explain the origin of BSE. The first proposes the spontaneous occurrence of BSE in cattle. The second proposes the emergence of BSE as a result of scrapie-bearing material having been fed to cows and a subsequent species barrier cross from sheep to cows. Although the origin of BSE remains a matter for debate, the cause of the resulting epidemic is widely accepted. The spread of BSE through British cattle was attributed to changes in rendering practices in the UK.

Rendering (meaning to reduce, convert, or melt down by heating) serves to recover and transform waste from the food industry. There are two types of rendering processes, edible, or fit for human consumption and non-edible, or not fit for human consumption. It is the rendering of inedible items such as animal carcasses and offal that ultimately become valuable protein sources like meat and bone meals that are fed to livestock. This transformation takes place by crushing the carcasses etc. into smaller parts to improve cooking efficiency followed by cooking at high temperatures ranging from 121°C to 135°C for 15 to 180 min. Through this process waste material is transformed into marketable animal meal (CFIA, Risk Assessment of BSE in Cattle in Canada, <http://www.inspection.gc.ca/english/sci/ahra/bseris/bserise.pdf>).

The rising cost of fuel, and a drop in the price differential between meat and bone meal and tallow, dictated the elimination of the step in which tallow was extracted. This

extraction of fat was carried out using an organic solvent and required a high-energy input (Nathanson et al, 1997). It is suggested that extraction with hydrocarbon organic compounds served to denature the infectious BSE proteinaceous agent in the past. The elimination of this step, in addition to the practice of feeding meat and bone meal to cattle as a protein source, produced the conditions required for the resulting BSE epidemic. Cattle carrying the disease were rendered and became part of a meat and bone meal bearing the infectious BSE agent which was subsequently fed to other cattle as a protein source: by this route BSE was able to spread rapidly among British livestock. Whole cattle herds were incinerated in order to stop the spread of the disease.

The aetiological agent and avenues of transmission of the disease are also widely accepted although no indisputable evidence exists to support this. BSE has been identified as a prion “proteinaceous infective particle” disease (Prusiner, 1982). The protein thought to be involved is a ubiquitous normal host glycoinositol phospholipid (GPI) anchored sialoglycoprotein. This prion protein has been designated PrP with the normal form referred to as the cellular prion protein PrP^c , and the abnormally folded form scrapie prion protein designated PrP^{Sc} (as it was first identified in sheep)(Jackson and Collinge, 2001). In TSEs the normally soluble, proteinase-sensitive PrP^c becomes an insoluble, proteinase-insensitive PrP^{Sc} (Horwich and Weissman, 1997), as a result of a shift in protein conformation. The PrP^c protein, made up of primarily helical folds, shifts to the PrP^{Sc} version of the protein which is comprised primarily of beta sheets. Infectivity results from the PrP^{Sc} form of the protein having the ability to transform normal PrP^c to the PrP^{Sc} form. There are no other post translational or genetic differences between the

normal and aberrant forms of the PrP protein (Jackson and Collinge, 2001). The mechanism through which PrP^{Sc} protein induces conversion of PrP^c remains unclear, as is the cellular function of PrP protein. (Jackson and Collinge, 2001).

Prion diseases present a challenge to the scientific community since the mechanism through which prions effect disease remains beyond our current understanding. Despite a significant international research effort, it is still not known exactly how BSE originated, the biological function of native PrP^c, the exact mechanism by which PrP^{Sc} converts native PrP^c, or how this converted protein then manifests into tissue specific central nervous system disease. PrP^c protein resides in tissues other than the brain, though only brain tissue seems to exhibit disease. Studies in mice have shown that deletion of the PrP^c gene and the absence of the PrP^c protein from mice does not seem to translate into any significant biological deficiencies (Horwich and Weissman, 1997). In addition, attempts to use PrP^{Sc} to infect mice lacking PrP^c have resulted in the absence of the disease manifestation; this result is not surprising since the aberrant form requires the presence of sufficient quantities of the normal PrP^c that may undergo a conformational change in order to accumulate lethal levels of plaque.

In order to prevent the spread of BSE in the UK, the Bovine Spongiform Encephalopathy Order prohibiting the feeding of ruminant-derived protein to ruminants was put into effect on July 18th 1988 (BSE Inquiry Report <http://www.bseinquiry.gov.uk/files/lb/102/tab01.pdf>). Through these measures cattle not incubating the disease should not contract BSE through exposure to contaminated feeds.

In turn, cattle that do not contract the disease should not pose a risk to human health. In response to the global emergence of BSE, other countries which were not immediately affected have subsequently implemented their own bans on feeding ruminant materials to ruminants.

In September 1997, the Canadian government introduced an amendment to the Health of Animals Acts, commonly referred to as the Feed Ban, which prohibits the feeding of mammalian meat and bone meal to ruminants (including higher risk species such as cattle, sheep, goat, elk and deer) with the exceptions of horse and pig (CFIA 2005, Feed Ban Review

<http://www.inspection.gc.ca/english/anima/feebet/rumin/revexa/revexae.pdf>). Some ruminant products such as skim milk powder, blood meal and tallow are considered low risk and therefore are not included in the ban. Material from equine, porcine, fish and poultry are permitted in feeds to be fed to ruminants.

Recently, three domestic BSE cases and one North American case have been reported and confirmed. The first case was detected in May of 2003, the second in December 2003 in Washington State that was imported from Canada, and the third and fourth in January of 2005 (CFIA 2005, Feed Ban Review

<http://www.inspection.gc.ca/english/anima/feebet/rumin/revexa/revexae.pdf>). In three of the four cases imported feed or tainted feed consumed prior to the ban is suspected due to the age of the affected cows (CFIA BSE in North America <http://www.inspection.gc.ca/english/anima/heasan/disemala/bseesb/bseesbindexe.shtml>).

The first cow in 2003 was determined to be 6-8 years of age, the second cow was born April 9th, 1997 and the third case Oct 1996. The last case however was a cow born March 1998 following the institution of the ruminant feed ban (CFIA BSE in North America <http://www.inspection.gc.ca/english/animas/heasan/disemala/bseesb/bseesbindexe.shtml>). In all four cases transmission of BSE through feeding of infected imported or domestic material is suspected. Even for the last post- ban case, feed is thought to be the method of transmission due to a special formulation fed to the cattle on that particular farm (CFIA BSE in North America <http://www.inspection.gc.ca/english/animas/heasan/disemala/bseesb/bseesbindexe.shtml>).

The emergence of these Canadian cases produced the same public and political concern that followed the UK epidemic. Borders were closed, consumer confidence in the safety of consuming beef declined and billions of dollars were lost to the Canadian beef industry. Because no native cases of BSE had been reported, and then four cases arose within a relatively short time period the emergence of the disease has been attributed to feeding with contaminated feed. There are differing opinions regarding the cause of the Canadian cases. CFIA describes feed imported or produced prior to the ban as the likely cause, while consumers fear that Canadian feed restrictions may be inadequate since the ages of the afflicted cows coincide with the introduction of the feed ban. Perhaps contamination and subsequent transmission through feeds, in spite of the feed ban, are potentially responsible for current BSE cases. Only recently, in December 2004, was specified risk material (spinal chord tissue) required to be removed from cattle carcasses (CFIA, New Regulations Proposed for BSE-related Feed Controls

<http://www.inspection.gc.ca/english/corpaffr/newcom/2004/20041210e.shtml>). Prior to December 2004, mistakes made during rendering, such as the inclusion of a cow carcass from the wrong lot or accidental feeding of the wrong feed to cattle, may have resulted in exposure of cattle to contaminated rations.

To restore consumer confidence in Canadian beef, the feed industry must implement measures that stand up to public scrutiny. A comprehensive feed testing program demonstrating compliance with the feed ban is necessary. Information on the precise nature of animal species which may be present in feeds constitutes an important element of any risk based approach to verify compliance with the regulatory regime.

In order to successfully determine compliance of manufactured feeds with the Canadian ban, individual animal feeds should be tested at the rendering, feed mill and farm lot levels. There are three major technologies that are recognized for their ability to identify contamination of ruminant feeds: microscopy, immunoassay and amplification of species-specific DNA sequences. The techniques vary greatly in cost and methodology as well as the type of information produced and are based on microscopic, protein, or DNA analysis.

1.0 Microscopy

The most widely accepted method for verifying compliance is by microscopic analysis. The European Union uses microscopy as the official method for testing samples for monitoring of their feeding ban, as described in the Commission Directive 2003/126/EC on the analytical method for the determination of constituents of animal

origin for the official control of feedstuffs. Microscopy is very effective in identifying the presence of minute amounts of meat and bone in a given sample.

In Canada, microscopic samples are floated in chloroform to divide the sample into two fractions: a light fraction made up of organic material that floats on top of the chloroform, and a heavy fraction consisting of bone and minerals that sink to the bottom of the chloroform. Each fraction is in turn separated by large and fine particle size (for a total of four fractions) and analyzed using stereo and compound microscopes. A stereoscope is used for the analysis of larger particle sized material (Figure 1.0).

Stereoscopic analysis allows the identification of components that make up the main ingredients of a feed, or the identification of contaminants (unintentional) or adulterants (intentional) present at high levels. Fine particles of each fraction are analyzed under a compound microscope that allows magnifications of 100-200 X (Figure 1.1 and 1.2).

Figure 1.0- Bovine Meat and Bone Meal viewed using a stereoscope. Using the magnification allowed by a stereoscope it is only possible to distinguish hairs in the field of view from the 'meat meal fragments'. These meal fragments are a combination of meat and bone particles.

Photo courtesy of Neil Vary - CFIA

Figure 1.1 –Bone fragments viewed with a compound microscope at 100X magnification. At this magnification it is possible to see qualities of the sample fragments, such as lacunae (air spaces) characteristic of bone.

Photos courtesy of Neil Vary – CFIA

Figure 1.2 - Striated muscle tissue viewed using a compound microscope at 200X magnification. At this magnification it is possible to see the striation features of the tissue characteristic of muscle.

Photos courtesy of Neil Vary – CFIA

At the magnification allowed by a compound microscope it is possible to identify minute fragments of animal bone and muscle tissue as well as hair and hydrolysed feather fragments and fish scales. The ability to distinguish these tissues is important when trying to determine the presence or absence of banned material. Ingredients such as hydrolysed feathers and fish scales are potential indications of a non-ruminant, non prohibited protein source.

By viewing the four fractions of a given sample, an experienced analyst can by observation detect very small amounts of animal material in a sample, and estimate the level of contamination. However, this method does not allow for the determination of the species origin of any meat or bone tissue identified. For this reason the European Union has banned all animal derived proteins except fishmeal, hydrolysed proteins and dicalcium phosphate (a mineral made from bone) from being fed to livestock. These regulations are outlined in Commission Regulation 1234/2003. It does state in the Commission document that porcine and poultry feed pose little risk and should be used in animal feeds but the inability to distinguish porcine and poultry derived proteins from ruminant proteins prevents this.

The lack of information pinpointing the species from which a tissue originated makes it difficult to confirm whether a sample is in direct violation of the feed ban. In addition, the processing of a single sample by an experienced microscopist takes on average about 4 hrs per sample. Operating a national testing program would require many well trained microscopists using many microscopes to evaluate enough samples to

constitute a representative number of field samples per year. Considering Canada possesses 29 rendering plants, only six of which process prohibited and non-prohibited material, 550 feed mills, 94 of which handle prohibited material and manufacture ruminant feed (CFIA 2005, Feed Ban Review <http://www.inspection.gc.ca/english/animal/feebet/rumin/revexa/revexae.pdf>) and an undefined number of livestock farmers, it is evident that the number of potential samples to be subjected to a comprehensive national testing program would be significant.

The training of microscopists is also an issue as it can take a significant amount of time to acquire the required skills, ranging from months to years. Most of the results obtained by a microscopist are subject to the personal judgement of that individual, and this can create variability in information on a specific sample.

1.1 Enzyme-Linked Immunosorbent Assay

Another technology for testing feeds uses immunoassay approach such as the Enzyme-Linked Immunosorbent Assay (ELISA) technique (Figure 1.3). Antibodies raised against a target antigen (a substance that can trigger an immune response) are fixed to a surface, which is incubated with a sample of interest (Janeway et al, 1999). If the target antigen is contained in this sample, it is bound by the fixed antibodies and unreacted material is washed away. A subsequent incubation with antigen specific antibodies bearing an enzyme label enables detection of the captured antigen by assaying bound enzyme (Janeway et al, 1999). The label-bearing antibody will only bind if the

antigen is present in the screened sample and therefore detection of the label is only possible in samples that contain the antigen of interest.

Proteins are molecules (antigens) recognized by antibodies. Proteins are macromolecules composed of sequences of specific amino acids. Amino acid composition determines the folding and therefore conformation of proteins. As a result of folding, different antigenic determinants are present on a protein. These areas are recognized by different antibodies and are referred to as epitopes (Janeway et al, 1999). There are two types of antibodies, monoclonal and polyclonal. Monoclonal antibodies are specific to a single epitope on an antigen and polyclonal antibodies are a mixture of antibodies recognizing different epitopes of an antigen (Janeway et al, 1999). At present, there are a number of ELISA assays applicable to feed, manufactured privately and sold as kits. Most of the kits currently available in North America use monoclonal antibodies raised against Troponin I, a heat stable muscle protein (Chen and Hsieh 2002). These monoclonal antibodies recognize only ruminant species.

Examples of commercially available immunoassay kits are those sold by Neogen (Lansing, MI) and Elisa-Tek (Gainesville, FL) utilizing monoclonal antibodies raised against Troponin I. Neogen markets two kits, one that identifies ruminant material in a meat and bone meal with a claimed detectability of 2% ruminant material in an animal meal, and a second that identifies ruminant materials in a animal feed with a claimed detectability of 1% ruminant material in the feed. Neogen describes their assay principle (Neogen Reveal kit insert) as a single-step lateral flow immunochromatographic assay.

Figure 1.3- ELISA schematic.

Essentially this is comprised of a dip stick where the extract is wicked through a reagent zone, which contains antibodies specific for ruminant by-products conjugated to coloured particles. If ruminant by-product is present, it will be captured by the conjugated antibodies. The ruminant-antibody-particle complex is then wicked onto a membrane which contains a zone of antibody specific for ruminant by-product. This zone captures the complex allowing the particles to concentrate and form a visible line (Neogen Reveal kit insert).

The ELISA-Tek Ruminant Species Kit claims the ability to detect ruminant Meat and Bone Meal (MBM) in non-ruminant meat and bone meals at $<0.1\%$ and in feeds at $<1.0\%$. The ELISA-Tek kit uses the ELISA microtiter plate format described in Figure 1.3. Colour change in a plate format is measured by absorbance with a microtiter plate reader. It is therefore possible to set a cut off absorbance value to differentiate between a positive and negative result. Although the ELISA techniques are similar the results are evaluated by different processes.

The use of ELISA technology to monitor compliance of the ban in Canadian feeds has several benefits and drawbacks. Some of the benefits include ease of use, quick sample turnaround, and target specificity. Furthermore, directions and necessary components are packaged in a kit that requires little training to use. One analyst can test multiple samples at the same time, which is amenable to high testing throughput. Also, because ELISA targets proteins tests can be designed to detect only proteins present in

muscle tissue that is banned, but not blood or milk, which are allowed as feed components.

Drawbacks of ELISA technology for this application include limited sensitivity, and some issues stemming from its reliance on protein identification described in the following section. ELISA kits such as those previously described (Neogen) claim a 1-2% detectability for MBM in another matrix. When looking at the amount of feed cattle can ingest, a one percent contamination level can translate into a significant amount of ingested prohibited material. Studies done in the UK have shown oral doses of 1g of infected material to be sufficient to transfer disease, and current studies have been expanded to include challenges with doses as low as 1mg of infected material (<http://www.defra.gov.uk/corporate/publications/pubcat/cvo/1999/chapter2.pdf>).

In feed testing, it is necessary for the antibody target to be a heat stable protein or epitope. Heat stability is necessary because meat and bone meals are processed at high rendering temperatures (120°C-133°C). ELISA testing kits frequently claim to identify 1% Meat and Bone Meal (MBM) in a feed. The amount of meat, in a meat and bone meal, is variable. Meat and bone meals are generally comprised of 30-40% bone and 60-70% protein. The protein portion consists of tissues such as skin, hair and muscle. An ELISA system designed to target muscle proteins will detect meat and bone meal with varying degrees of accuracy. Establishing an ELISA's capability to detect MBM at specific levels is difficult, as these assays are only detecting the muscle portion of the

MBM (Chen and Hsieh 2002). Studies qualifying a detection limit are difficult to carry out due to the variability in MBM content and processing.

The last difficulty with the ELISA revolves around the species specificity of the reagent antibodies. Tests are geared towards identifying a protein from a single species like bovine, or a protein that is common to many species (i.e. ruminants). To process a sample for the detection of all the different ruminant species covered by the ban, it would be necessary, to use ELISAs specific to each species (i.e. several tests) or a single ELISA that recognizes a protein common to all ruminants (i.e. those kits discussed previously). To date individual ELISAs do not exist for the different ruminant species encompassed by the ban.

1.2 Polymerase Chain Reaction

As an alternative to the other methods, the Polymerase Chain Reaction (PCR) technique has been used to target and amplify animal species-specific DNA sequences. DNA is targeted with complementary primers and amplified many times to generate enough amplicon to be visible when stained and resolved on an agarose gel.

There are three basic steps involved in a PCR reaction: denaturation, annealing and elongation. Each of these steps these are repeated for 25-40 cycles. Denaturation at 94°C, separates the double stranded DNA into two single strands and also serves to stop enzymatic reactions from previous cycles. Annealing at 52-65 °C, facilitates primers attaching to the complementary sequence on the single strands. During the subsequent

elongation at 72 °C, Taq DNA polymerase attaches at the sites of primer binding and starts copying the single stranded template; polymerization continues until the nascent strand is completed, or until the next round of denaturation (Figure 1.4).

Conventionally after all the desired cycles of amplification are complete the product is processed by loading an aliquot onto an agarose gel alongside an appropriate molecular size marker, passing an electrical current through the gel to separate amplification products by size. Following which the products may be visualized by staining with ethidium bromide and exposure to UV light.

A PCR method targeting a mitochondrial DNA sequence that includes the ATPase subunit 8 and the amino-terminal portion of the ATPase subunit 6 protein, unique to bovine has been developed for the detection of tissues from this priority species in feeds (Tartaglia et al, 1998), although the presence of other ruminant species of interest cannot be ascertained using this approach.

A feature of interest with the above PCR is the use of a mitochondrial DNA target. Mitochondrial DNA is targeted because it is present in high copy number in eukaryotic cells (Myers et al, 2001), and its sequence can be highly conserved within a species. The rendering process to which animal carcasses are subjected can be quite damaging to cellular components, including DNA. Average rendering conditions in Canada range from 120-132°C for 15-120 min.

Figure 1.4 – PCR schematic

One plant that does processing by batch cooking even uses 155°C for 180 min (CFIA

Risk Assessment of BSE in Cattle in Canada

<http://www.inspection.gc.ca/english/sci/ahra/bseris/bserise.pdf>). These rendering conditions can severely damage or cleave DNA in tissues. By using a short mitochondrial DNA sequence (271 bp) as a target sequence, the probability of finding undamaged sequence to amplify is much greater. Using a short sequence increases the probability of an intact fragment being present to amplify, and using high copy number mitochondrial DNA as a target increases the number of potential targets for amplification (Myers et al, 2001).

Two other PCR systems that target conserved mitochondrial DNA sequences for the detection of multiple animal species have been described (Saulle et al, 1999 and Bellagamba et al, 2001). The first of these PCR systems targets a conserved region of the cytochrome B gene among vertebrates (Bellagamba et al, 2001). The second system targets a region within the *tRNA^{Lys}* and *ATPase8* genes of primarily in ruminants (also horse and pig) (Saulle et al, 1999), and was demonstrated to amplify DNA from a broad variety of species (Myers et al, 2003). An attractive feature of using either of these approaches is the use of a single primer pair targeting sequences common to different animal species. The cytochrome B target method referred to as the “vertebrate” PCR, successfully amplified DNA from animal species bovine, buffalo, sheep, goat, horse, pig, chicken and turkey, which are likely to occur in North American feeds (Bellagamba et al, 2001). The *tRNA^{Lys}* and *ATPase8* target method referred to as the “universal” PCR method successfully amplified DNA from the principal ruminant species (bovine, sheep,

goat, elk and deer) also likely to occur in North American feeds as well as DNA from horses and pigs (Myers et al, 2003).

For both of these methods restriction endonuclease analysis is required to distinguish amplicons arising from prohibited and exempted materials (Bellagamba et al, 2001 and Myers et al, 2003). A multiplex PCR approach in which several target DNA sequences were co-amplified using multiple primer pairs in a single reaction has also been developed for the identification of different animal species (ruminants, poultry, fish and pork) in feeds (Dalmaso et al 2004). In all of the preceding methods, the analysis of amplicons generated by PCR is achieved on the basis of their differential electrophoretic mobility by agarose gel electrophoresis. Agarose gel electrophoresis analysis is time-consuming and the complex electrophoretic patterns obtained with multiplex PCR systems can be difficult to interpret. Furthermore, the identity of individual amplicons can be difficult to confirm, especially if restriction endonuclease analysis is required.

This thesis describes the development of a simple low density array technique based on the use of a macroporous, hydrophobic polyester cloth containing immobilized DNA probes as a solid support for the detection and characterization of PCR amplicons by hybridization. The advantages of polyester cloth as a solid phase for DNA array hybridizations have previously been demonstrated (Blais et al, 2002). Polyester cloth possesses a macroporous, hydrophobic surface which allows immobilization of a wide variety of capture molecules including proteins, nucleic acids and lipids by passive

absorption (Blais and Yamazaki, 1997). Once fixed with UV, this cloth-capture matrix presents a high affinity absorbent environment for specific analytes, which may subsequently be detected by immunoenzymatic methods (Blais and Yamazaki, 1997). The macroporosity and thickness of the cloth as compared to other matrices such as nitrocellulose and nylon allows absorption of larger sample volumes per unit of area (Blais and Yamazaki, 1997). Yet another advantage of its macroporosity is the ease and speed of washing between reaction steps to remove unbound reagents. Polyester cloth is also cost-effective and yields improved reaction kinetics and shorter hybridization times due to its large and readily accessible surface. Polyester cloth serving as a favourable matrix for the immobilization of DNA probe arrays followed by subsequent hybridization and detection of multiplex PCR products has previously been described for the characterisation of bacterial pathogens in foods (Gauthier and Blais, 2004) and referred to as a Cloth-Based Hybridization Array System (CHAS).

This thesis addresses the development of a CHAS for the detection and identification of animal species of concern in feeds. For this purpose the development of an array of oligonucleotide probes designed to be specific for species-variable sequences in the universal amplicon will be examined. The principle animal species addressed include bovine, sheep, goat, elk, deer which represent higher risk ruminant species commonly kept as livestock in the agricultural industry. Bovine are known to suffer from BSE, sheep (to which goat is closely related) from scrapie, and elk and deer from chronic wasting disease.

In preparation for the following assay, the vertebrate (Bellegamba et al, 2001) and universal (Myers et al, 2003, Saulle et al, 1999) PCRs were carried out with each ruminant species (bovine, sheep, goat, elk and deer) individually and the amplification products for each were sequenced for the purpose of designing oligonucleotide (oligo) capture probes specific to each species. Sequencing of the vertebrate PCR products was not possible and further development of the CHAS proceeded using only the universal PCR. In the proposed CHAS, amplicons incorporating digoxigenin-labelled dUTP (dig-dUTP) are generated with the universal PCR (Myers et al, 2003, Saulle et al, 1999), followed by hybridization of the amplicons with an array of animal species-specific oligonucleotide probes immobilized in discrete spots on a polyester cloth strip, and immunoenzymatic detection of the bound amplicons using anti-DIG antibody peroxidase conjugates. The scheme for this approach is illustrated in Figure 1.5.

Another objective of this thesis is to demonstrate the performance characteristics of the CHAS in the detection of prohibited ruminant-derived materials in feeds. It is proposed that the CHAS will be a valuable adjunct to other techniques such as microscopy in the analysis of feeds for the presence of prohibited ruminant-derived materials.

Figure 1.5 - Cloth Hybridization Array System schematic.

Development of the CHAS and Optimization of Conditions

Chapter Two

Materials and Methods

Design and Optimization

Objective: As described in the preceding chapter the goal of this work was to develop a combined PCR and detection technique to identify ruminant material in animal feeds. In order to accomplish this goal the first necessity was to find or design primers to identify the targets of interest. The first set of primers identified in the literature, designated 'vertebrate' primers, described the ability to detect all vertebrates including, bovine, buffalo, sheep, goat, horse, pig, chicken and turkey (Bellagamba et al, 2001). Subsequently, a second set of primers was identified in the literature (Myers et al, 2003) that described the amplification of DNA from ruminant species such as bovine, sheep, goat, elk and deer, these primers were designated 'universal' primers. Experimentation had begun using only the vertebrate primers but once identified, the merit of also using the universal primers was realized and experimentation continued with both sets.

It was necessary to optimize the vertebrate PCR for sufficient amplification. Optimization of the PCR required testing individual parameters such as buffer composition, annealing temperature and evaluation of the resulting enhancement or decrease in amplification of the target.

Finally it was necessary to optimize all of the steps of the CHAS procedure in terms of sensitivity and specificity for maximal detection. As with the PCR, optimization of the CHAS assay was performed by testing individual parameters such as hybridization duration and temperature as well as probe concentration to evaluate which conditions provided the maximum signal.

The following sections describe the optimization of each part of the PCR–CHAS assay.

2.0 Primers, PCRs and DNA Extraction

The vertebrate specific primer set Vertebrate Forward - 5'

CCATCCAACATCTCAGCATGATGAAA, and Vertebrate Reverse - 5'

GCCCCTCAGAATGATATTTGTC amplify a 359 bp region in all vertebrate species

(Bellagamba et al, 2001). The ruminant specific universal primer set Universal Forward -

5' ACTTTGAAAAATGATCTGCATCAA and Universal R - 5'

TCGTTCATTTTGTCTCAAGGGGT amplify a 271 bp region in all ruminant species

including pig (Saulle et al, 1999 and Myers et al, 2003). These primer sequences were

synthesized by Sigma Genosys.

In order to verify the performance of the afore mentioned PCR systems genomic DNA targets from different animal species were required. Blood samples from bovine, sheep, elk, red deer, pig and goat were collected by Canadian Food Inspection Agency veterinarians from local farms. DNA from each sample was extracted using the Wizard Genomic DNA Purification Kit (Promega A1120) with the protocol for Isolation of Genomic DNA from whole blood, and a sample size of 300 µl. Each sample was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and produced the following DNA concentrations: bovine, 10 ng/µl; pig, 32 ng/µl; red deer, 4 ng/µl; elk, 22 ng/µl; sheep, 14 ng/µl; and goat, 18 ng/µl.

2.1 Vertebrate PCR Conditions

PCR reactions using the vertebrate primers and 100-200 ng of genomic DNA from bovine, pig, red deer, elk, sheep and goat (Bellagamba et al, 2001) were performed according to the conditions detailed by Bellagamba et al. In a 50 µl reaction, RedTaq 1X reaction buffer, 0.8 mM of dNTPs, 15 pmol of each primer, and 2.5 units of RedTaq Polymerase with the exception of substituting Hotstar Taq Polymerase (Qiagen, Cat #203203) (which requires a 15 min warm up at 94 °C) for RedTaq Polymerase. The amplification program detailed in the publication of Bellagamba et al, 2001 is as follows: 1 min at 94°C, followed by 5 sec at 94°C, 30 sec at 50-55°C, 40 sec at 72°C for 20-30 cycles followed by a 2 min 72°C final extension cycle. This cycling program was followed, with the exception of a 4 °C hold at the end of the program and an additional 15 min warm up required by the Hotstar Taq Polymerase. No amplification product was visible on a 1.2% agarose gel for any of the species tested.

The vertebrate primers were then assayed with a different PCR reaction mixture formula and cycling program previously used in OLC (Ottawa Laboratory Carling). Final concentrations in a 50 µl reaction were 10 mM Tris HCl (pH 8.3) 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP (Sigma DNTP-100), 0.4 µM each vertebrate primer, 0.2 µg/ml BSA, 2 units of Taq Polymerase (Sigma D4545). 5 µl of each template (bovine, pig, red deer, elk, sheep and goat) as detailed for this PCR was used resulting in a range of 20-160 ng/rxn of template depending on the species tested (genomic concentrations from blood extractions for each species are detailed above). The amplification program was as follows; 3 min at 80°C, 1 min 30 s at 94°C, then 1 min at 94 °C, 1 min at 58 °C, 1

min at 72 °C repeated for 29 cycles, followed by 1 min at 94 °C, 30 sec at 58 °C and 10 min at 72 °C and a hold at 4 °C. Faint amplification product was visible for bovine and goat (Figure 3.0).

2.2 Buffer Optimization

To enhance amplification pH and MgCl₂ were optimized in a buffer checkerboard using the preceding PCR recipe with 2.5 units HotStar Taq polymerase. HotStar Taq was substituted solely because it was available and the Taq of choice in other endeavours, and it had been substituted globally in the laboratory for other Taq Polymerases. The numbers 1 through 12 assigned in the checkerboard below represent the pH and MgCl₂ concentrations that intersect at that box (i.e. #1 would represent a pH of 8.3 and a MgCl₂ concentration of 3 mM) and correspond to the number designations in Figure 3.1.

pH and MgCl ₂ concentration of optimization buffers				
Tris.HCl pH	MgCl ₂ Concentration			
	3 mM	3.5 mM	4 mM	4.5 mM
8.3	1	2	3	4
8.5	5	6	7	8
8.8	9	10	11	12

The 50 µl reaction contained either 50 ng of bovine DNA, 90 ng of goat DNA or 70 ng of sheep DNA (these amounts represented 5 µl of stock as detailed by the method of Myers et al, 2003). The amplification program was 15 min at 94°C, then 1 min at 94°C, 1 min at 65°C and 1 min at 72°C for 29 cycles followed by a last cycle of 1 min at

94°C, 1 min at 65°C and 10 min at 72 and then a 4°C hold. The amplification products were visible on a 1.2% agarose gel (Figure 3.1).

2.3 Annealing Temperature

Using the most successful conditions, Tris-HCl pH 8.3 and 4.4 mM MgCl₂, a gradient PCR using annealing temperatures ranging from 54.4-66.0°C was performed using a Eppendorf Mastercycler Gradient. Each different temperature was assigned a corresponding number, to allow identification with the sample tested at that temperature.

1 – 54.5 °C	4 – 56.3 °C	7 – 60.5 °C	10 – 64.6 °C
2 – 54.7 °C	5 – 57.5 °C	8 – 62.0 °C	11 – 65.5 °C
3 – 55.3 °C	6 – 59.0 °C	9 – 63.4 °C	12 – 66.0 °C

In each reaction 5 µl of either bovine, sheep, pig, goat, red deer or elk DNA was used to determine the best annealing temp for the reaction. The most favourable temperature for the reactions appeared to be 57°C (Figures 3.2 and 3.3).

2.4 Optimized PCR Conditions

The final PCR recipe used for a 50 µl reaction of either primer set was;

50 mM Tris, 0.02 µg/µl BSA 4.5 mM MgCl₂,
 200 µM each dNTP,
 0.4 µM each primer,
 2.5units of Hot Star Taq
 5 µl of DNA sample (concentration of DNA variable).

The final cycling program was;

94°C for 15 min
 94°C for 1 min
 57°C for 1 min
 72°C for 1 min
 Repeated for 29 cycles
 94°C 1 min
 57°C for 1 min
 72°C for 10 min
 Hold at 4°C.

The ruminant primers were identified in the literature as the optimization of the vertebrate primer set neared completion. The ruminant universal primers were assayed using the same PCR reaction mixture detailed above as well as the same amplification program with the exception of a 52°C annealing temp as described in the paper of Myers et al, (2003) and demonstrated good amplification. No further optimization was carried out for this set of primers.

2.5 Preparation of genomic DNA from meat samples

Beef (*Bos taurus*), elk (*Cervus elaphus*), pig (*Sus scrofa*), sheep (*Ovis aries*) and goat (*Capra hircus*) meats were collected at a local slaughter house by Canadian Food Inspection Agency (CFIA) staff. Chicken (*Gallus gallus*), buffalo (*Bison bison*), salmon (*Salmon salar*), and horse (*Equus caballus*) meats were purchased from local retailers. White tailed Deer (*Odocoileus virginianus*) meat was a gift from a local sportsman. Meats were cut into small cubes, frozen and ground with mortar and pestle in liquid nitrogen, then stored at -20°C until use. Note: at this point white tailed deer was used as opposed to red deer due to its greater prevalence in the Canadian market. Corn (*Zea*

mays) and soybean (*Glycine max*) genomic DNA was prepared as previously described (Blais et al, 2002). DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20°C until use.

Several different commercial kits were examined in an attempt to obtain maximal yields of genomic DNA.

(1) The first method used for isolating genomic DNA from ground tissues was carried out with the Invisorb Forensic Kit I (Invitek Cat No.10341003). Approximately 20 mg of each tissue was weighed out and extracted as per kit protocol 9: DNA Isolation from tissue sample. DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20°C until use.

(2) The second method for isolating genomic DNA from ground tissues was done using the Wizard Genomic DNA Purification Kit. (Promega A1120). 15-20 mg of each tissue was weight out and extracted using the protocol for animal tissues. The tissue preparation was done for 40 min at 65°C and the lysis and protein precipitation was done for 30 min at 37°C. DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20°C until use.

(3) The third method for isolating genomic DNA from ground tissues was carried out using the Wizard Plus Minipreps DNA Purification Kit (Promega) according to Lipp *et al.* (1999). 300 mgs of each meat sample was processed. DNA was quantified using a

Hoefer DyNA Quant 200 Fluorometer (Amersham Biosciences) and stored at -20°C until use.

Following extraction, DNA from all eleven species was amplified using the Vertebrate and Universal primer sets. Reactions for both sets of primers were done using 10ng of genomic DNA.

2.6 PCR Specificity and Sequencing

Once it was determined that each primer set recognized the species it claimed (Table 3.0), (all species for Vertebrate primer set, and all species except chicken and salmon for Universal primer set) additional reactions were carried out to produce enough amplicon for sequencing using 10 ng of genomic DNA per reaction. For each set of primers one reaction containing corn, and one containing soy as well as a reaction mixture only tube were included as controls.

Amplicon generated from each species, using with each primer set was pooled and purified using Qiagen PCR purification kit (Cat no. 28104), and sent to GENEWIZ (New Jersey) for sequencing.

2.7 Oligonucleotide Probe Design

Sequences were not resolvable for any of the vertebrate samples. The DNA sequences from the universal amplicons of the principal ruminant species of interest (bovine, sheep, goat, elk, deer) were aligned using DNAMAN software (Lynnon

Corporation). Regions of sequence variability were identified for the preparation of species-specific oligonucleotide capture probes (Table 3.1). Oligonucleotide probes were synthesized by a commercial contractor (Sigma Genosys) for each of the principal ruminant species (bovine, sheep, goat, elk and deer) and upon reception were re-hydrated to 100 μ M concentrations with 1X TE buffer.

2.8 Final PCR for the CHAS

To test the CHAS detection system a PCR was carried out using the Universal primer set with the reaction conditions detailed above with the inclusion of 5 μ M DIG-11-dUTP (Roche) per reaction. The cycling program was identical as detailed above for the Universal PCR reactions.

2.9 Incubation of Probes on the Polyester Cloth for the Cloth-based Hybridization Array System (CHAS)

The CHAS assay was attempted using 100 μ l PCR reactions, with the same final concentrations of each reagent as was used in the 50 μ l PCR reactions described previously. Reactions were performed bearing 0 ng, 1 ng, 10 ng of bovine DNA per reaction. The CHAS procedure prior to optimization was as follows. Polyester cloth (DuPont, Sontara 8100) was cut into 1.5 $\times$ 5 cm strips, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips were then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M MgCl_2 and 0.15 M NaCl] and lightly blotted immediately prior to use for oligonucleotide probe immobilization. The oligonucleotide probes (1 μ M final concentration) were

diluted in coating buffer and 100 μ l was heated at 100 °C for 10 min (to disrupt any possible secondary structures), then placed on ice. Probes (5 μ l) were pipetted in discrete spots on a cloth strip, followed by incubation at 37 °C for 30 min. Probes were cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 mJ/cm²) using a UVP cross-linker (Model Stratalinker 1800, Stratagene). The strips were blocked by incubating for 1 h at 37 °C with hybridization solution [5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they were washed five times by saturating strips with 0.01 M phosphate-buffered saline [pH 7.2/0.15 M NaCl (PBS)] containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips were air-dried and stored at 4 °C until use.

2.10 Cloth-based Hybridization Array System (CHAS)

Conditions prior to optimization. For the assay DIG-labelled universal PCR products, amplicons were denatured by heating at 100 °C for 10 min, and mixed with 900 μ l of ice-cold hybridization solution containing 50% (v/v) formamide. The entire mixture (1 ml) was pipetted onto a strip of probe-coated cloth and incubated for 30 min at 45 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations were carried out at room temperature. Strips were saturated with 1 ml of anti-DIG-peroxidase conjugate (Roche) diluted 1:2000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips were saturated with 1 ml tetramethylbenzidine (TMB) membrane peroxidase

substrate (Kirkegaard and Perry Laboratories), and incubated for 30 min. Reactions were graded qualitatively as follows: positive (blue spot), negative (no spot).

2.11 Final Optimized CHAS Conditions

Following optimization the final CHAS conditions were identical to those detailed above with the following exceptions; optimal oligo probe concentration was determined to be 10 μ M, optimal hybridization time was determined to be 10 min and an optimal anti-dig POD concentration was determined to be 1:1000.

Chapter 3

Results and Discussion

Design and Optimization

3.0 Results for the Vertebrate PCR

The first attempt to amplify vertebrate DNA (deer, pig, bovine, sheep, goat, elk) using the vertebrate primers with the conditions and cycling program detailed in the literature (Bellagamba et al, 2001) produced no visible amplification product at all.

The second attempt to amplify DNA from bovine, sheep, goat, elk, deer and pig using the vertebrate primers and PCR reaction mixture and cycling program used regularly for a bovine PCR in the laboratory produced faint amplicon bands for some animal species (Figure 3.0). Faint bands can be seen in lanes four, six and twelve corresponding to amplification having taken place for bovine and goat genomic DNA. The faint bands are seen corresponding to the 369 bp marker and since the expected amplification product is 359 bp, these faint bands seemed to correspond to the expected amplicon size. As amplification was somewhat successful using the last set of conditions, it confirmed amplification using the vertebrate primers was feasible and that likely this reaction needed to be further optimized.

3.1 Optimizing pH and MgCl₂ - Buffer Checkerboard

The first step in the optimization of this PCR was to estimate melting temperatures of the vertebrate primers as it may affect optimal annealing temperature. It was of interest to compare how close the theoretical calculated annealing temperature was to the annealing temperature (55°C) in the published conditions (Bellagamba, 2001) that produced no amplification, and the annealing temperature used in the preceding

somewhat successful amplification attempt (58°C) (Figure 3.0). Melting temperature was calculated as follows; $T_m = 2^{\circ}\text{C} \times (\text{A}+\text{T}) + 4^{\circ}\text{C} \times (\text{G}+\text{C})$

Calculated T_m for the Vertebrate Forward Primer = 74°C and for Vertebrate Reverse Primer = 76°C.

The guideline for estimating annealing temperature uses the calculated melting temperature minus 5°C (Promega Hot Star Taq PCR Handbook). The estimated annealing temperatures would be 69°C and 71 °C for the forward and reverse primers respectively. These calculations produced extremely high predicted annealing temperatures.

Because the first published protocol (Bellagamba et al, 2001) called for a 50-55°C annealing temp and the second protocol that produced some amplification used a 58°C annealing temp, it was decided to use an annealing temperature near the higher limit, 65°C, for annealing temperatures but well below the calculated value which appeared too high.

This change in annealing temperature combined with the buffer checkerboard produced the following amplification (Figure 3.1).

Figure 3.0 –PCR products amplified using vertebrate primers run on an agarose gel faint bands are visible in lanes 4, 6 and 12.

Figure 3.1 – Optimization checkerboard for pH and Magnesium concentration using vertebrate primers.

Bovine (B), Goat (G), and Sheep (S) DNA genomic DNA were used as targets with a 65°C annealing temperature in the cycling program. The numbers 1 through 12 correspond to pH and MgCl₂ concentrations assaying in the buffer checkerboard detailed in Materials and Methods Section 2.2. A 123bp molecular weight marker was loaded into Lanes 1 and 40.

Bands seen on the gel (Figure 3.1) show good amplification for all the buffers assayed when sheep or goat DNA was used. The bands resulting from the reactions that used bovine DNA were faint. The best results among the bovine reactions was visible using buffer 4 which contained 50 mM Tris pH 8.3, MgCl_2 4.5 mM and 0.02 $\mu\text{g}/\mu\text{l}$ BSA in the final reaction. This buffer was chosen to be used in ongoing experiments.

3.2 Annealing Temperature Gradient

After choosing a buffer to use, an annealing temperature gradient was performed using the vertebrate primers in order to further optimize the PCR reaction. Several annealing temperatures had been assayed to date and it was difficult to gauge the result of the different annealing temperatures on the basis of success of the amplification, so a gradient PCR was performed to provide clarity. In addition, porcine DNA was used in this attempt as opposed to the goat DNA used in the buffer checkerboard. Goat and sheep DNA both amplified well using the buffer checkerboard and bovine did not. As goat and sheep are very closely related phylogenetically it was possible that their success in amplification was somehow due to their similarity. It is not known why the bovine template amplified poorly as using vertebrate primers should result in the recognition of all vertebrate species equally. An alternate different third species, porcine, was chosen to use during optimization as it seemed prudent to use very different genomic targets to gauge any potential effect of species specific enhanced or decreased amplification.

Figure 3.2 – PCR annealing temperature gradient using vertebrate primers and Bovine (B), Sheep (S), or Porcine (P) DNA.

The number designations 1 through 12 correspond to different annealing temperatures to which the individual PCR reactions were subjected as detailed in Section 2.3 of the Material and Methods. A 123 bp molecular weight marker was loaded into Lanes 1 and 40.

Figure 3.3 - PCR annealing temperature gradient using vertebrate primers and Goat (G), Deer (D), or Elk (E) DNA.

The number designations 1 through 12 correspond to different annealing temperatures to which the individual PCR reactions were subjected as detailed in Section 2.3 of the Material and Methods. A 123 bp molecular weight marker was loaded into Lanes 1, 14 and 27.

Figure 3.2 showed successful PCR amplification for bovine, sheep and pig DNA. The same PCR and amplification conditions were repeated for elk, deer and goat Figure 3.3 in order to confirm and compare that the optimal annealing temperature would be similar for all the vertebrate species for which it was possible to obtain blood and subsequently extract genomic DNA. Figures 3.2 and 3.3 showed successful PCR reactions using DNA from all six species, which indicated that previously the poor amplification seen for the bovine DNA during the buffer optimization was more likely due to a pipetting error of the genomic DNA than any discrepancy between different species and the ability of the vertebrate primers to recognize them.

The optimal annealing temperature for the six species of interest appeared to be at gradient number 5, which corresponds to an annealing temperature of 57.5°C. From this point forward the annealing temperature for the PCR cycling program was set at 57°C when amplifying product using the vertebrate primers.

Following optimization of the vertebrate PCR, and identification of the existence of the universal primer set, the same reaction mixture and amplification conditions (save a 52°C annealing temp as detailed in Myers et al 2003) were tested with the universal primer set successfully (Figure 3.4). Because good amplification with the universal PCR was achieved using the optimal conditions for the vertebrate reactions further optimization of the universal reactions was considered unnecessary.

Figure 3.4 – DNA from Ruminant Species Amplified by the Universal Primers Using the Optimized PCR Conditions.

PCRs were performed using 10 ng of genomic DNA from each of the species with the optimized PCR reaction conditions detailed in Section 2.4. A 123 bp molecular weight marker was loaded into Lanes 3.

Upon determining the success of the universal PCR reactions it was necessary to test the specificity of each primer for a wider range of targets. Up to this point all of the genomic DNA used had been harvested from blood samples collected by agency veterinarians. Blood samples were unobtainable for many of the species of interest. Therefore, meat from each species of interest was collected, and DNA was extracted and used in subsequent experiments.

3.3 Extraction of Genomic DNA from Meat Samples

As extracting genomic DNA is more difficult from connective tissue it was necessary to grind the tissue with liquid nitrogen prior to extraction.

Three different commercially available extraction kits were compared for the extraction of DNA from the ground samples. The Invitek and Promega Genomic extraction kits produced poor yields when quantified by fluorometer. The yields using the first two kits ranged from 0 to 14 ng/ μ l. The third method examined using the Promega Wizard Kit and provided much higher yields of DNA, 250-500 ng/ μ l.

3.4 Species Recognition

Both the vertebrate and universal primers were used with the optimized PCR and amplification parameters and contained 10 ng of DNA from each species to test whether the primer sets amplified what they claimed. The resulting amplifications are summarized below (Table 3.0).

Table 3.0. Amplification of species specific DNA using Vertebrate and Universal PCR systems.

Species	Vert Rxn	Univ Rxn
Bovine	+	+
Sheep	+	+
Goat	+	+
Elk	+	+
Deer	+	+
Buffalo	+	+
Horse	+	+
Pig	+	+
Chicken	+	-
Salmon	+	-
Corn	-	-
Soybean	-	-

This table summarizes the results of agarose gel electrophoresis analysis performed using DNA from each of the species specified. A “plus” symbol indicates the presence of amplicon, and a “minus” symbol indicates the absence of amplicon.

Each primer set did recognize the expected range of species. The vertebrate primers recognized all vertebrates tested and the universal ruminant primers recognized all the ruminants tested as well as pig, as claimed. It is important to note that plant species such as the soybean and corn tested were not amplified either PCR system. This is important because these are main grain ingredients of many animal feeds.

After completion of several amplifications for each species using each primer set the PCR products for each species were pooled, purified and sent for sequencing. The sequencing reactions failed for all samples amplified with the vertebrate primer set. Chromatographs of the sequence information showed more than one population of amplicon existed in each of the reactions. It was therefore not possible to determine a single sequence for any of those samples.

The universal samples were all sequenced successfully with the exception of the pig sample which suffered from the same problem as the vertebrate samples. Sequencing was not possible as more than one amplification product was evidently present in the reaction.

The failure of all of the vertebrate and the pig universal samples in sequencing was surprising. Prior to shipping an aliquot from each pooled product to be sequenced was loaded and separated on an agarose gel. In all cases the aliquot run appeared as one band of the expected size, and for this reason each sample sent for sequencing was thought to be a pure uniform sample. It is not known for the samples that failed

sequencing whether there was a major product visible on the gel and a minor product present in small quantity not resolvable by agarose gel electrophoresis, or whether the amplicons present in each failed sample had different sequences but the same overall amplicon size and therefore co-migrated on a gel and appeared to be one band.

In designing this assay it was necessary to choose a manageable number of species to be included on the cloth strip. Five species were chosen, those most prevalent as food sources or in the agriculture industry, and aligned using DNAMAN software. A 32-mer species specific region was identified for each. The following oligo sequences were synthesized corresponding to that variable region for use as probes in the CHAS (Table 3.1).

3.5 CHAS Assay

Once these oligonucleotides were synthesized, a preliminary CHAS assay was performed to see if hybridisation between PCR product and fixed oligonucleotide would be successful. PCR reactions containing 10 ng, 1 ng and 0 ng of bovine DNA as well as dig dUTP were carried out in duplicate. Oligonucleotides were spotted onto the polyester cloth in the following order bovine, elk, sheep, deer and goat (Figure 3.5). The spotting pattern obtained for 1 ng and 10 ng PCR reactions upon completion of the CHAS assay is shown in Figure 3.5. A spot at the site of bovine oligonucleotide fixation shows a successful assay. Despite the success of the proposed CHAS system the weak appearance of the spot indicated that the assay required optimisation to intensify the hybridization reactions. For the optimisation the bovine detection system was used as the model. All

PCR reactions were carried out using bovine DNA as template and all cloths were spotted with bovine oligonucleotide probe.

3.6 CHAS Optimization of Hybridization Time

Optimization began by altering the length of time hybridization was allowed to progress as well as the amount of DNA added to the PCR reactions (Table 3.2). The initial hybridization times examined were 30 min, 60 min, 90 min and 120 min. The results for this first assay were not as expected. The 30 min hybridization provided the best results and as hybridization duration increased signal decreased and then disappeared entirely. Generally hybridizations are more efficient longer hybridization times and the shortest time that provides a good signal is used to facilitate a given assay (Roche Applied Science Typical Hybridization Times, http://www.roche-applied-science.com/PROD_INF/MANUALS/DIG_MAN/dig33-37.pdf). Roche even suggests that for oligonucleotide:DNA hybrids that the hybridization time must be at least 1-6 hr and up to overnight.

In order to further investigate these results, an assay including shorter hybridization periods was performed. Surprisingly, a 10 min hybridization period produced the best signal among the array incubation periods tested (Table 3.2).

Table 3.1. Partial universal amplicon sequences used for oligonucleotide probe design.

Species	Nucleotide Sequence
Bovine	5' AAAAGTTTCAAAACACAACCTTTTATCACAATCCAGAAGT <u>GACACCAACAAAA</u> 3'
Sheep	5' AAAAATCTCAAAACACAACCTTCTACCACAACCCAGAATTAATAACAACAAAA 3'
Goat	5' AAAAATCTCAAAGTACGACTTCTACCACAACCCAGAATTAA...CAGCAAAA 3'
Elk	5' AAAAATTTCGAAACACAATTTTCACTTTAATCCAGAAGT <u>AGTATCAACCAAAA</u> 3'
Deer	5' AAAAGTTTCAAAACATAATTTTTCTTTAATCCAGAACCTACATCAACTAAAA 3'

Universal amplicons obtained with genomic DNA from different ruminants were sequenced and aligned to identify regions of variability for oligonucleotide probe design. Partial sequences of the relevant areas for the different species are shown. Gaps in the sequence are indicated by dots (...). Probe sequences are indicated by underscoring.

Figure 3.5 – Example CHAS results from the first universal PCR-CHAS assay attempt using bovine DNA.

Probes on the array were as follows B, bovine; E, elk; S, sheep; D, deer; G, goat.

It was speculated that a secondary structure may form in the denatured amplicon causing interference with hybridization, and perhaps even resulting in displacement of hybrids by progressive intra-strand interaction. The oligonucleotide fixed to the cloth and used in the hybridization is 32-mer, which is short compared to the length of the 271 bp amplicon. The oligonucleotides are complementary to a section near one end of the amplicon strands (Figures 3.6). It is unknown how much of each oligonucleotide is available for hybridization with amplicons after UV cross linking to the polyester cloth, since a portion of the oligonucleotide probe is involved in tethering it to the cloth surface, so perhaps over time secondary structure forming along the single stranded amplicon is blocking the oligonucleotide binding site. To gauge potential inhibition by secondary folding the oligonucleotide probe binding sites were located within the 271-mer complementary amplicon strands and the potential for formation of secondary structures within each 271-mer amplicon was determined using the predict secondary structure feature of the DNAMAN software. An example of the predicted secondary structure for the 271-mer bovine strand can be seen in Figures 3.6.

Some secondary structure is predicted for most of the amplicons in the oligonucleotide probe binding sites. Using the bovine 271-mer amplicon as a model (Figure 3.6) if the structure was possible one third of the probe binding site may fold onto itself potentially blocking binding. Since secondary structure is only predicted to occur in a portion of the probe-complementary region, and given the AT-rich nature of the base pairing region, it is unlikely that such a structure would form during stringent hybridization conditions used in these experiments.

Table 3.2. Detectability of Bovine dig-labelled amplicon binding Bovine oligonucleotide probe for different hybridization times.

Species	DNA/Rxn (ng)	CHAS Reactivity (No. Positive/total) For different hybridization times in minutes					
		10	20	30	60	90	120
Bovine	10	++/++	+/+	+/+	+/+	-/-	-/-
	1	++/++	++/++	+/+	+/+	+/-	+/-
	0.1	++/++	+/+	+/+	+/+	+/-	+/+
	0	-/-	-/-	-/-	-/-	-/-	-/-
Index of reactivity:					/ separates each replicate

PCR reactions were performed using different amounts of bovine template. These PCRs was subsequently used in for a CHAS assay in which hybridization was allowed to precede for varying time periods in order to determine the optimal hybridization time period of the CHAS assay.

Hybridization between oligonucleotide probe and target does occur during the first 10 min. The decrease in signal observed over time suggests that oligonucleotide probe/single strand hybrid is progressively dissociating over time.

If secondary structure in the binding site on the single strand was occurring, it would be occurring during the first 10 minutes of the hybridization as well as throughout the extended hybridization period. Secondary folding might occur or not, and would depend on whether the nucleotides encompassed by the potential folding site came into close enough contact to bind each other. Even if the 'chance' of the involved bases coming into contact with each other was good there should still be instances where the configuration did not occur and secondary structure does not form. Using this scenario one would expect to see some minimal signal of oligonucleotide probe/strand hybrid formation for all the incubation periods assayed. In addition secondary structure formation potentially explains how formation of oligonucleotide probe/strand hybrids may be inhibited, but does not explain why hybrids that are formed during the first 10 min are somehow dissociated and not observed at longer incubation periods. Hybrids that have formed should not be lost. These results indicate that interference is not at the initial binding stage but rather, that formed hybrids are gradually dissociated. Formation of secondary structure does not seem a feasible as a cause for this phenomenon. It is believed that these results represent the re-annealing of the 271 bp complementary strands which results in the cleavage of the oligonucleotide probe-single strand hybrid.

The 271-mer target is made single stranded by heating to 100°C for 10 min. Once single stranded it is reacted with a 32-mer oligonucleotide capture probe. Binding occurs between the probe and the single stranded section of DNA it is complementary to. Because the probe is 32-mer both end sections of the 271-mer strand remain unbound. Floating all around in the hybridization solution are the single strands complementary to the pieces bound by the probes. If a complementary strand in solution finds a free end of a piece bound to a probe they will zip back together and pull the strand hybridized to the probe right off to complete re-annealing of a double stranded 271 bp amplicon. Therefore if the hybridization is stopped after 10 min and excess hybridization solution containing the free floating 271-mer complementary strands are washed away there is no competition for the strand bound by the probe and the oligonucleotide probe-amplicon hybrid stays intact and can be detected in the subsequent assay steps. But if hybridization is allowed to proceed for long periods of time, all of the 271-mer single stranded complementary strands re-anneal and the oligonucleotide probe-amplicon hybrids are disrupted as the 271-mer strands re-anneal. These double stranded amplicons would then be washed away with subsequent washes as they are not bound to the cloth and no signal is detected by the assay.

Not only is maximal signal development at 10 min a function of 'in comparison to' subsequent signal loss over longer incubation times but as previously described polyester cloth provides a large surface area that promotes rapid reaction kinetics that in turn results in very rapid initial binding. This means maximal binding after only 10 min.

Therefore allowing the hybridization to proceed for no more than 10 min is a critical control point in the assay.

3.7 Optimization of Capture Probe Concentration

To further optimize reaction conditions different concentrations of oligonucleotide probes spotted onto cloths were assayed. Different hybridization (10 and 30 min) were studied to verify that the 10 min hybridization time continued to be optimal when the capture probe concentrations were altered.

Results of the experiment indicated a higher concentration of probe than had been used to date (1 μM in previous experiments) would be optimal (Table 3.3). The 5 and 10 μM spots were close in intensity, and the 10 μM probe concentration was chosen for subsequent experiments. As demonstrated previously the 10 min hybridization produced more intense spots than the longer 30 min hybridization period for each probe concentration assayed.

3.8 Optimization of Hybridization Temperature

The next parameter tested was the effect of hybridization temperature (Table 3.4). A higher hybridization temperature increases stringency which is required to assure the specificity of hybridizations. This CHAS utilizes a 45°C hybridization temperature. Other similar CHAS procedures (Gauthier et al, in press) use a 37°C hybridization temperature. It was of interest to determine whether this CHAS assay would benefit from less stringent conditions by altering temperature.

Table 3.3. Detectability Bovine dig-labelled amplicon binding different concentrations of bovine oligonucleotide probe when hybridized for 10 or 30 min.

CHAS Reactivity (intensity/replicate)				
Hybridization (Mins)	Oligonucleotide Probe Concentration (μM)			
	1	5	10	15
10	+/+	++/++	++/++	++/++
30	-/-	+/+	+/+	+/+

Index of reactivity:

/ separates each replicate

Bovine DNA was amplified using the universal PCR. The amplicon was quantified and the CHAS assay was performed using 100 ng bovine amplicon/ml per cloth. The complementary bovine oligonucleotide probe was spotted at different concentrations on the polyester cloth.

It was possible that a less stringent hybridization temperature would allow formation of a larger number of oligonucleotide probe:DNA target hybrid and result in an increase in signal. When compared to cloths using the exact same conditions with a 45°C hybridization (Table 3.4), it was found that lowering the hybridization temperature to 37 °C reduced signal (Table 3.5). This was not expected, and it is speculated that the reduction in stringency may serve to enhance the phenomenon previously described, where the single stranded 271 bp amplicon re-anneals thereby separating the oligonucleotide probe:DNA hybrid. The 45°C hybridization temperature was therefore used for all subsequent experiments.

3.9 Optimization of Anti-Digoxigenin-Horseradish Peroxidase (POD)

Continuing optimization it was necessary to investigate altering parameters of the anti-dig POD labelled antibody detection components. The anti-dig antibody recognizes and binds to sites along the nucleic acid hybrid where digoxigenin was incorporated during the PCR. The antibody bearing the POD conjugate is in turn reacted with TMB to visualize sites of antibody attachment. The antibody incorporation is an integral step to the overall success of the assay and for this reason determining the effect of concentration of antibody used as well as duration of the anti-dig POD incubation with the cloth bound hybrids was a necessity. A more concentrated anti-dig antibody solution may increase the number of anti-dig antibodies binding the digoxigenin target which in turn would increase signal intensity. Likewise a longer incubation period could allow increased binding of the digoxigenin target by the antibodies in solution and in turn could potentially increase the intensity of the resulting signal.

Table 3.4. Detectability of Bovine dig-labelled amplicon binding different concentrations of bovine oligonucleotide probe at an alternate hybridization temperature when hybridized for 10 or 30 min.

Hybridization (Min)	CHAS Reactivity			
	Oligonucleotide Probe Concentration			
	(µM)			
	1	5	10	15
10	-/-	+/-	+/-	+/-
30	-/-	+/-	+/-	+/-

Index of reactivity:

/ separates each replicate

Bovine DNA was amplified using the universal PCR. The amplicon was quantified and the CHAS assay was performed using 100 ng amplicon/ml per cloth with hybridization at 37°C for 10 or 30 min. The complementary bovine oligonucleotide probe was spotted at different concentrations on the polyester cloth. And the hybridisation was performed at 37°C as opposed to 45°C used in all CHAS assays to date.

To date the anti-dig antibody was used at a 1:2000 dilution and incubated with the cloth for 10min. These conditions were compared to a 1:1000 anti-dig antibody concentration incubated for 10 and 30 min (Table 3.5). The 1:1000 dilution used did seem to enhance the assay signal intensity. The incubation time periods then were then tested over a larger time range in order to more clearly determine its effect on signal intensity (Table 3.6). A 10 min incubation period was determined to be as effective as longer periods.

3.10 Detectability of a Shorter Target Sequence

In the literature it has been suggested that targeting a shorter region for amplification in the sample DNA improves the detectability of PCR for highly processed samples (Gizzi et al, 2003). In a situation where the target DNA is subjected to harsh processing conditions such as high heat (e.g. rendering conditions) there is the potential for target DNA degradation. It is postulated that a shorter target sequence may have a better chance of surviving rendering and therefore be easier to detect in feeds. Taking into account the loss of signal seen during the assay when hybridization was extended past 10min it seemed prudent to attempt our assay with a smaller target sequence. As detailed in section 3.6, the free ends of the 271 bp strand bound by the oligonucleotide probe may begin to re-anneal with its free-floating partner thereby cleaving the oligonucleotide probe-DNA hybrid. If the length of the unbound ends of the probe bound strand were decreased susceptibility to finding and re-annealing to their single stranded mate may be decreased.

Table 3.5. Detectability of Bovine dig-labelled amplicon using a more concentrated anti-dig POD dilution.

Anti-Dig POD dilution	CHAS Reactivity	Incubation time with Anti-Dig POD (min)
1:1000	-/++	10
1:1000	++/++	30
1:2000	+/-	10

Index of reactivity:

/ separates each replicate

Dig-labelled bovine DNA was amplified using the universal PCR and subsequently 500 ng of amplicon per cloth was used in a CHAS assay. Cloths were spotted with 10 μ M concentrations of bovine oligonucleotide probe. Anti-dig antibody concentration was increased to determine the detectability of the assay when more antibody was used for the detection steps. A different concentration and two different incubation times were compared against the conditions currently utilized for the assay. A 1:1000 anti-dig POD dilution was shown to produce a more intense signal.

Table 3.6. Detectability of Bovine dig-labelled amplicon when varying anti-dig POD incubation times using a 1:1000 dilution of anti-dig POD.

Incubation time with Anti-Dig POD (min)	CHAS Reactivity
10	++/++
20	++/++
30	++/++
60	++/++
Index of reactivity:	/ separates each replicate

Dig-labelled bovine DNA was amplified using the universal PCR and subsequently 500 ng of amplicon per cloth was used in a CHAS assay. Cloths were spotted with 10 μ M concentrations of bovine oligonucleotide probe. Different incubation periods with anti-dig antibody were assayed in order to assess the affect of incubation period on signal intensity. A 10 min incubation period with anti-dig antibody was shown to be equally successful as longer incubation periods.

A different forward bovine primer designated Bovine Internal Forward–5'CATCTTTCAACTAAAAGTTTCAAA was synthesized and used in the PCR with the reverse universal primer and it was followed by a hybridization assay using the conditions determined to date to be optimal. The location of this forward primer in relation to the forward primer used previously can be seen in Figure 3.7. The amplicon produced was 138 bp and was confirmed by gel electrophoresis. Interestingly, the only difference seen in overall performance of the assay was the shift in optimal hybridization time period. (Table 3.7). Instead of peak signal intensity at 10 min hybridization the peak signal was at 30 min hybridization. Signal was still lost if hybridization was allowed to progress for a longer time period. This seems to indicate that shortening the free ends of the DNA bound by probe did decrease the potential for interaction with their free-floating mates and subsequent re-annealing. Given enough time however eventually the single stranded mates did come into contact and re-anneal as longer hybridization times still showed complete signal loss (Table 3.7).

There was no intensity gain in using a shorter amplicon sequence and in fact the overall time to perform an assay was increased by 20 min (The additional 20min of hybridization time). Designing a shorter amplicon for each species would have resulted in a multiplex PCR situation (5 different forward primers, and one reverse) as opposed to a universal primer situation (there were no areas of conserved sequence in the 271 bp amplicon that were common to all the species of interest that would allow universal primer design that would produce in a shorter amplicon). Overall, the idea of using

shorter target sequence was discarded, as there seemed to be no benefits and several drawbacks.

With respect to target degradation during rendering results presented later in this thesis (Table 5.3 and 5.4) using the universal PCR with DNA extracted from heat treated material showed no indication that target of 271 bp would be problematic as a result of template degradation. All samples tested displayed good recovery of our target. In addition it has been suggested that in those instances where target degradation was addressed as an issue for overall success assay it was in fact the intricacy of the extraction process with which the problem resided (Myers et al, 2005 in press). Target degradation has also been suggested to be an issue more related to European rendering conditions which typically occur at 133°C for 30 min, and even for this scenario studies have shown that amplicons 271 bp in size are reliably amplified and detectable after processing at these more stringent conditions (Tartaglia et al, 1998).

3.11 Species Specificity of the CHAS assay

To date the work performed has focused on optimizing CHAS parameters using the bovine detection system as a model. It was then necessary to establish the ability of the CHAS assay to differentiate between the species of interest (bovine, sheep, goat, elk and deer) amplified by the universal PCR. Towards this end and using the optimized CHAS conditions, each species was assayed with the CHAS.

Table 3.7. Detectability of a shorter DNA amplicon.

Time (min)	CHAS Reactivity
10	+/+
20	+/+
30	++/++
60	-/-
90	-/-
120	-/-

Index of reactivity:

/ separates each replicate

A 138 bp sequence was amplified using the universal PCR reaction conditions and the Bovine Internal Forward Primer and Reverse Universal Primer. The DIG-labelled amplicon was used in a CHAS assay with various hybridization incubation times. The optimal signal was shown to be when the hybridization was allowed to progress for 30 min.

Each assay showed the ability to bind the target species DNA with the appropriate oligonucleotide probe (Figure 3.8). There was some cross reactivity between the close phylogenetically related species goat and sheep (Figure 3.8). The sequences for the sheep and goat in the oligonucleotide probe region have only a four base pair difference (Table 3.1) and these differences are at either end of the probe, the internal bases are common to both species which is obviously a homologous enough sequence to allow cross-reactivity between the two species.

In an attempt to limit cross reactivity the hybridization assay was carried out for each species using various hybridization temperatures; 40°C, 45 °C, 50 °C and 55 °C to assess the effect of temperature on non-specific binding (Table 3.8). Cross reactivity between sheep and goat did decrease at the higher (more stringent) temperatures 50 °C and 55 °C, but overall signal decreased among the other species at the higher temperatures. The decrease in cross reactivity was not significant enough to warrant increasing the hybridization temperature given the consequent decrease in the signal obtained for the other species (Table 3.8).

Figure 3.8 – Spotting pattern of the cloth assay for each species using the universal PCR.

From top to bottom the species DNA added to the PCR subsequently used for the CHAS was bovine, sheep, goat, elk, deer. The probes are in the order bovine, B; sheep S; goat G; elk E; deer D and are spotted in duplicate on each cloth. This cloth was taken with a digital camera and the colours are exactly as they appear on a bench top assay. It is possible to see the cross reactivity that occurs between sheep and goat.

Table 3.8. Detectability of amplicon using different hybridization (stringency) temperatures.

Species	Hybridization Temp (°C)	CHAS Reactivity (intensity/replicate)				
		B	S	G	E	D
Bovine	40	++/++	-/-	-/-	-/-	-/-
	45	++/++	-/-	-/-	-/-	-/-
	50	+/+	-/-	-/-	-/-	-/-
	55	+/+	-/-	-/-	-/-	-/-
Sheep	40	-/-	++/++	++/++	-/-	-/-
	45	-/-	++/++	++/++	-/-	-/-
	50	-/-	++/++	+/+	-/-	-/-
	55	-/-	++/++	+/+	-/-	-/-
Goat	40	-/-	++/++	++/++	-/-	-/-
	45	-/-	++/++	++/++	-/-	-/-
	50	-/-	+/+	++/++	-/-	-/-
	55	-/-	-/+	++/++	-/-	-/-
Elk	40	-/-	-/-	-/-	++/++	-/-
	45	-/-	-/-	-/-	++/++	-/-
	50	-/-	-/-	-/-	+/+	-/-
	55	-/-	-/-	-/-	+/+	-/-
Deer	40	-/-	-/-	-/-	-/-	++/++
	45	-/-	-/-	-/-	-/-	+/++
	50	-/-	-/-	-/-	-/-	+/+
	55	-/-	-/-	-/-	-/-	+/+

Index of reactivity:

/ separates each replicate

Genomic DNA from each species was amplified using the universal PCR. The PCR products were quantified and 500 ng of dig-labelled amplicon was used per cloth in the CHAS assay. Hybridizations for each species of interest with cloth bearing probes for the five species of interest (bovine, sheep, goat, elk, deer) were carried out at 40°C, 45°C, 50°C and 55°C in order to determine the effect of temperature on cross-reactivity.

3.12 Final Optimized CHAS conditions

The final optimised CHAS conditions were as follows;

Cloth Preparation

Polyester cloth (DuPont, Sontara 8100) is cut into 1.5×5 cm strips, and washed with 95% (v/v) ethanol, followed by rinsing with deionized distilled water on a filter with vacuum suction. The strips are then soaked in coating buffer [0.1 M Tris/HCl (pH 8.0), 0.01 M MgCl_2 and 0.15 M NaCl] and lightly blotted immediately prior to use for oligonucleotide probe immobilization. The oligonucleotide probes (10 μM final concentration) are diluted in coating buffer and 100 μl is heated at 100 °C for 10 min (to disrupt any possible secondary structures), then placed on ice. Probes (5 μl) are pipetted in discrete spots on a cloth strip, followed by incubation at 37 °C for 30 min. Probes are cross-linked to the cloth by exposing the strips to UV light for 1 min (254 nm, 100 mJ/cm^2) using a UVP cross-linker (Model Stratalinker 1800, Stratagene). The strips are blocked by incubating for 1 h at 37 °C with hybridization solution [$5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine and 1% (w/v) protein blocking reagent (Bio-Rad)], after which they are washed five times by saturating strips with 0.01 M phosphate-buffered saline [pH 7.2/0.15 M NaCl (PBS)] containing 0.05% (v/v) Tween 20 (PBST) on a filter under vacuum suction. The resulting probe-coated cloth strips are air-dried and stored at 4 °C until use.

Cloth Hybridisation Assay

Denaturation by heating at 100 °C for 10 min of the 100 µl dig-labelled universal PCR reactions, and mixing with 900 µl of ice-cold hybridization solution containing 50% (v/v) formamide. The entire mixture (1 ml) is pipetted onto a strip of probe-coated cloth and incubated for 30 min at 45 °C, followed by washing with PBST on a filter under vacuum suction. All subsequent incubations are carried out at room temperature. Strips are saturated with 1 ml of anti-DIG-peroxidase conjugate (Roche) diluted 1:1000 in PBST containing 0.5% (w/v) protein blocking reagent (Bio-Rad) and incubated for 10 min. After washing with PBST, the strips are saturated with 1 ml tetramethylbenzidine (TMB) membrane peroxidase substrate (Kirkegaard and Perry Laboratories), and incubated for 30 min. Photos are taken of the cloths to document the results of the assay.

Evaluation of the Performance Characteristics of the CHAS

Chapter 4

Materials and Methods

Performance Evaluation

4.0 Parameters for the Evaluation

Once all the conditions of this assay were optimised it was necessary to evaluate the performance characteristics of this assay. For a qualitative method such as this one the following performance characteristics should be validated: target reactivity, target matrix, detectability, sensitivity, specificity, the number of false negatives and the number of false positives. Several different target types were used in establishing this validation including; genomic DNA extracted from meat which was processed at 121°C for 20 min for each species, DNA extracted from simulated feeds described below and DNA extracted from externally supplied samples.

Target reactivity – refers to the determination of the reactivity of the method with target and non-target analytes. An example as applied to this assay would be the ability of the assay to detect ruminant DNA in an extraction aliquot containing the ruminant DNA as well as various other non-target DNAs.

Target Matrix – refers to the composition of the sample containing the analyte. For this assay this would be the grain based animal feeds.

Detectability – refers to the ability of the target method to detect different levels of target analyte. As applied to this method it would be the ability of the method to detect serial dilutions of genomic DNA or different levels of meat meal in the simulated meat samples.

Sensitivity – Is the ability of the assay to detect the analyte when it is present. As applied to this assay it would be the number of true positives and true negative results.

Specificity – refers to the ability of the method to recognize a specific target.

With respect to this assay it would be the ability of the assay to distinguish between the species amplified by the universal primers.

The number of false negatives and positives – refers to any samples misidentified by the assay. A false negative refers the inability to identify the analyte when it is in fact present in a sample, and a false positive refers to the detection of the analyte when it is not present in the sample.

The following experimental protocols served to test these parameters.

4.1 Genomic DNA samples

Genomic DNA extracted as per Section 1.5 from all eleven vertebrate species and two plant species were assayed by the combined PCR-CHAS. (Table 5.0).

Genomic DNA from each species of interest (bovine, sheep, goat, elk, deer) extracted as per Section 2.5 were serially diluted from 100 pg/μl – 50 fg/μl. 10 μl of each dilution was used in the subsequent universal PCR reactions for detecting various amounts of genomic DNA.

4.2 Preparation of simulated meat meal-in-feed blends

Soybean meal and Beef Chop (consisting of corn, barley and molasses) were purchased from local retailers. The absence of animal by-products in these feeds was ascertained by microscopic examination for the presence of meat and bone particles, as well as by the universal PCR CHAS assay. The feeds were mixed together in the

proportion of 20% Soybean Meal and 80% Beef Chop, and then processed in a Robocoup R6VN blender (Ridgeland) until a fine powdery consistency was achieved. This sample comprised the blank feed matrix (BFM) into which simulated meat meals were subsequently blended.

Simulated meat meals (SMM) from each of the principal ruminant species were prepared individually by cutting the meats into small cubes, followed by cooking at 121°C in an autoclave (1 atmosphere) for 30 min in order to simulate rendering conditions. The cooked meats were then frozen at -80 °C and freeze-dried for ease of subsequent grinding. These samples were ground with a mortar and pestle to achieve a fine particle size resembling the consistency of commercial meat meals.

Simulated meat meal-in-feed blends were prepared gravimetrically by blending SMM into BFM at defined levels (5.0, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.001% [w/w]) as follows: to make a 5.0 % (w/w) blend, 2.5 g of SMM were added to 47.5 g of BFM in a 250 mL-capacity screw cap container, followed by mixing in a Turbula Mixer (model T2F, Glen Mills Inc.) for 30 min. Blends containing lower levels of simulated meat meal were prepared by successive serial dilutions of blend with BFM and mixing as above.

4.3 Externally acquired feed samples

Samples of lactating dairy feed fortified with 0.1% (w/w) of either bovine meat-and-bone meal, lamb meal or porcine meat-and-bone meal (all commercially rendered)

were kindly provided by M. Myers, Center for Veterinary Medicine, United States Food and Drug Administration.

4.4 Extraction of DNA from feed samples

Total DNA was extracted from feed samples using buffers and reagents supplied in the Invisorb Forensic Kit 1 (Invitek) according to a procedure developed by M. Myers (pers. comm.). Briefly, 0.25 g of feed sample was mixed with 1.0 ml of Lysis Buffer D and 200 μ l Bone Lysis Enhancer with vigorous vortexing. The samples were placed in a 60 °C waterbath for 2 h, and then centrifuged at 13,200 rpm for 2 min. The supernatant was recovered and mixed with 15 μ l of silica suspension and 250 μ l of Lysis Buffer D. Each sample was vortexed for 10-15 sec and incubated at room temperature for 5 min, followed by centrifuging at 10,000 rpm for 10 sec. The supernatant was discarded, and the silica pellet was washed once with Lysis Buffer D and twice with wash buffer. For each wash the silica pellet was resuspended by vortexing and then centrifuged for 10 sec at 10,000 rpm. After the last wash, the supernatant was discarded and the silica pellet dried by placing the open tube in a 60 °C heat block for 5-10 min, followed by resuspension in 70 μ l Elution Buffer, incubation at 60 °C for 5 min, and centrifugation at 13,200 rpm for 2 min. About 60 μ l of the resulting supernatant was transferred to a clean tube, and subjected to a final centrifugation step to ensure removal of any residual silica. This final supernatant containing the DNA was removed to a clean tube and stored at -20°C until use.

Following extraction, of all of the simulated meat mixes and externally acquired samples, were subjected to the CHAS assay.

Chapter 5

Results and Discussion

Performance Evaluation

5.0 Specificity

Once the PCR and CHAS assay had been optimized all the species for which DNA had been extracted were tested by the assay in order to verify its specificity (Table 5.0). All of the species were identified as expected, with phylogenetically closely related species showing cross reactivity including buffalo and bovine, and sheep and goat (Table 5.0).

Upon determination that the assay recognized the different species accurately it was necessary to test whether the assay would recognize multiple species when present in the same reaction (Figure 5.0). As it is a possibility that several species may be present in a sample, it was necessary to determine the capability of this assay to detect several species at once. All of the combinations of species that were tested produced the expected results. In each case each species was detected if added to the original PCR reaction (Figure 5.0).

5.1 Detectability and Sensitivity

At this point the species specificity of the assay was demonstrated as well as the ability to identify each species of interest when numerous species were present in a mixture. Additional investigations proceeded to test the detectability and sensitivity of the combined PCR-CHAS for each species using purified genomic DNA. The detectability of the assay for the different animal species was in the range of 0.1-1.0 pg (Table 5.1) and the sensitivity of the following assays showed the assay capable of reliably detecting the species when present with the exception of the cross reactivity of the sheep and goat.

Table 5.0. Reactivity of universal PCR products in the CHAS

Species	Amplicon	CHAS Reactivity (No. positive/total)				
		B	S	G	E	D
Bovine	+	2/2	0/2	0/2	0/2	0/2
Sheep	+	0/2	2/2	2/2	0/2	0/2
Goat	+	0/2	2/2	2/2	0/2	0/2
Elk	+	0/2	0/2	0/2	2/2	0/2
Deer	+	0/2	0/2	0/2	0/2	2/2
Buffalo	+	2/2	0/2	0/2	0/2	0/2
Pig	+	0/2	0/2	0/2	0/2	0/2
Horse	+	0/2	0/2	0/2	0/2	0/2
Chicken	-	0/2	0/2	0/2	0/2	0/2
Salmon	-	0/2	0/2	0/2	0/2	0/2
Corn	-	0/2	0/2	0/2	0/2	0/2
Soy	-	0/2	0/2	0/2	0/2	0/2

Genomic DNA (1.0 ng) from different animal species was subjected to the combined universal PCR-CHAS procedure as described in Methods. PCR product was also subjected to agarose gel electrophoresis analysis to ascertain the presence (+) or absence (-) of the ca. 271 bp universal amplicon. CHAS reactivity with the different probes on the array is expressed in terms of the number of positives per total number of replicate samples tested. Probes were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

Figure 5.0. Detection of various combinations of different animal species.

Different combinations of genomic DNA (1 ng each) from various animals were subjected to the combined universal PCR-CHAS procedure. Combinations of genomic DNA for strip A: bovine, sheep, goat, elk and deer; for strip B: elk and goat; and for strip C: sheep and deer. Each probe spot was applied to the polyester cloth strips in duplicate. Probes on the array were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

Once the detectability of the assay was shown for each species it was necessary to show the detectability of the assay for a species when in the presence of an excess of DNA from another species amplified in a single PCR (Table 5.2). This scenario was designed to mimic a sample in which ruminant material from two different species were present in the sample in different quantities (i.e. one in excess of the other). It was necessary to qualify whether detectability of the species present in the minority would be affected (Table 5.2). This showed potential interference when one species in the assay is present in a vastly greater quantity than another. The ability to reliably detect bovine DNA decreased from 0.1 pg to 5 pg (Table 5.2) when in the presence of excess sheep DNA. The detection of minute amounts of one species DNA appears to be inhibited by the presence of excess amount of another, likely due to increased chance of priming occurring in the more prevalent species as the target sequences are common to both species. The universal primers would likely be more apt to encounter and subsequently bind the template DNA present in greater quantity in the PCR reaction.

5.2 Target Reactivity and Target Matrix

The preceding experiments were performed using purified genomic DNA dilutions. Real samples would require extraction of DNA prohibited material from a grain-based feed. Samples composed of a tissue-grain combination may present challenges for efficient DNA extraction as well as show PCR inhibit from matrix effects. It was necessary to examine the performance of the method with actual feed samples.

Table 5.1. Detection of various amounts of genomic DNA

Species	DNA (pg)	CHAS Reactivity (No. Positive/total)				
		B	S	G	E	D
Bovine	5.0	2/2	0/2	0/2	0/2	0/2
	1.0	2/2	0/2	0/2	0/2	0/2
	0.5	2/2	0/2	0/2	0/2	0/2
	0.1	2/2	0/2	0/2	0/2	0/2
Sheep	5.0	0/2	2/2	2/2	0/2	0/2
	1.0	0/2	2/2	2/2	0/2	0/2
	0.5	0/2	2/2	1/2	0/2	0/2
	0.1	0/2	0/2	0/2	0/2	0/2
Goat	5.0	0/2	2/2	2/2	0/2	0/2
	1.0	0/2	2/2	2/2	0/2	0/2
	0.5	0/2	0/2	2/2	0/2	0/2
	0.1	0/2	0/2	0/2	0/2	0/2
Elk	5.0	0/2	0/2	0/2	2/2	0/2
	1.0	0/2	0/2	0/2	2/2	0/2
	0.5	0/2	0/2	0/2	2/2	0/2
	0.1	0/2	0/2	0/2	0/2	0/2
Deer	5.0	0/2	0/2	0/2	0/2	2/2
	1.0	0/2	0/2	0/2	0/2	2/2
	0.5	0/2	0/2	0/2	0/2	0/2
	0.1	0/2	0/2	0/2	0/2	0/2

Different quantities of genomic DNA from various ruminant species were subjected to the combined universal PCR-CHAS procedure. CHAS reactivity with the different probes on the array is expressed in terms of the number of positives per total number of replicates tested. Probes were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

Table 5.2. Detection of bovine DNA in the presence of excess sheep DNA.

Bovine DNA (pg)	CHAS Reactivity (No. Positive/total)				
	B	S	G	E	D
100	2/2	2/2	2/2	0/2	0/2
50	2/2	2/2	2/2	0/2	0/2
10	2/2	2/2	2/2	0/2	0/2
5.0	2/2	2/2	2/2	0/2	0/2
1.0	1/2	2/2	2/2	0/2	0/2
0.5	0/2	2/2	2/2	0/2	0/2
0.1	0/2	2/2	2/2	0/2	0/2

Different quantities of bovine genomic DNA were mixed with a fixed amount (1 ng) of sheep genomic DNA and subjected to the combined universal PCR-CHAS procedure.

CHAS reactivity with the different probes on the array is expressed in terms of the number of positives per total number of replicates tested. Probes were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

In order to mimic real samples, simulated meat meal mixes were prepared and tested using the assay to evaluate the sensitivity of the assay for each species of interest when present in a grain mix (Table 5.3).

The meat was processed according to typical rendering conditions (heated to 121°C for 20 min) and the feed was composed of ingredients typically found in a ruminant grain based feed. For the prepared samples the grain was finely ground which introduces even more difficulty into the extraction process. Finer ground particles allow more surface area for lysis and subsequent DNA release. This would result in the release of more extraneous plant DNA along with the ruminant DNA in the sample. Whole grain real life samples would not be ground, rather sifted for finer material and these ‘fines’ would be tested. In a whole grain feed the contamination is mixed in with and between the grains. Contaminating material is only incorporated into the feed matrix in a pelleted feed where ground plant material has been pressed together. In a pelleted sample the pressed pieces would be disrupted back apart to release prohibited material potentially pressed into the pellet during manufacturer. The mixed samples therefore reflected the more difficult testing situation presented by a pelleted feed.

It was possible to detect meat products for all the animal species by percent weight at levels of 0.001-0.01 % in the mixed samples (Table 5.3). The assay therefore appeared to achieve a good detectability and target reactivity when target matrix was taken into account. Although to date a tolerance for contamination with ruminant products in feeds has not been determined.

Finally, the CHAS procedure was tested against samples prepared for a similar study by the Center for Veterinary Medicine, United States Food and Drug Administration. The PCR CHAS reliably detected and correctly identified the samples containing bovine or sheep material and showed no reaction with samples containing pig or those devoid of animal material (Table 5.4). The information in Table 5.3 and 5.4, demonstrate the absence of any false positive or false negative results produced by this assay.

The performance characteristics; target reactivity, target matrix, detectability, sensitivity, specificity, the number of false negatives and the number of false positives, of this qualitative method have been demonstrated.

Table 5.3. Detection of simulated meat meal blended in feed.

Species	Samples % blend (w/w) ($\times 10^{-2}$)	CHAS Reactivity (No. Positive/total)				
		B	S	G	E	D
Bovine	5.0	3/3	0/3	0/3	0/3	0/3
	1.0	3/3	0/3	0/3	0/3	0/3
	0.1	3/3	0/3	0/3	0/3	0/3
Sheep	5.0	0/3	3/3	3/3	0/3	0/3
	1.0	0/3	3/3	3/3	0/3	0/3
	0.1	0/3	2/3	2/3	0/3	0/3
Goat	5.0	0/3	3/3	3/3	0/3	0/3
	1.0	0/3	3/3	3/3	0/3	0/3
	0.1	0/3	1/3	1/3	0/3	0/3
Elk	5.0	0/3	0/3	0/3	3/3	0/3
	1.0	0/3	0/3	0/3	3/3	0/3
	0.1	0/3	0/3	0/3	3/3	0/3
Deer	5.0	0/3	0/3	0/3	0/3	3/3
	1.0	0/3	0/3	0/3	0/3	3/3
	0.1	0/3	0/3	0/3	0/3	0/3

Simulated meat meals from different ruminant species were blended with a grain-based animal feed as described in Materials and Methods. Total DNA was extracted from the blends and subjected to the combined universal PCR-CHAS procedure. CHAS reactivity with the different probes on the array is expressed in terms of the number of positives per total number of replicates tested. Probes were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

Table 5.4. Analysis of feed samples.

Blend	Sample	CHAS Reactivity (No. Positive/total)				
		B	S	G	E	D
Bovine	1	3/3	0/3	0/3	0/3	0/3
MBM	2	3/3	0/3	0/3	0/3	0/3
(0.1% [w/w])	3	3/3	0/3	0/3	0/3	0/3
Sheep	4	0/3	3/3	3/3	0/3	0/3
MM	5	0/3	3/3	3/3	0/3	0/3
(0.1% [w/w])	6	0/3	3/3	3/3	0/3	0/3
Porcine	7	0/3	0/3	0/3	0/3	0/3
MBM	8	0/3	0/3	0/3	0/3	0/3
(0.1% [w/w])	9	0/3	0/3	0/3	0/3	0/3
None	10	0/3	0/3	0/3	0/3	0/3
	11	0/3	0/3	0/3	0/3	0/3
	12	0/3	0/3	0/3	0/3	0/3

Total DNA was extracted from sample blends containing either meat-and-bone meal (MBM) or meat meal (MM), and then subjected to the combined universal PCR-CHAS procedure. CHAS reactivity with the different probes on the array is expressed in terms of the number of positives per total number of replicates tested. Probes were as follows: B, bovine; S, sheep; G, goat; E, elk; D, deer.

Conclusion

6.0 Conclusions

This thesis described the development of a cloth-based hybridization for the rapid detection and identification of PCR products derived from trace quantities of the ruminant species - bovine, sheep, goat, elk and deer - in a feed.

The broad reactivity of the universal PCR observed in this study confirms the earlier findings of Myers *et al.* (2003), who found it necessary to distinguish between banned and exempted animal species by restriction endonuclease analysis of the amplicons. As an alternative approach, I have demonstrated the applicability of the CHAS as a rapid and simple method for differentiating between universal PCR amplicons originating from different animal species. While some cross-reactivity was observed with respect to hybridization of amplicons with the probe array, in every instance the non-specific reactions appeared to correlate with a high degree of phylogenetic relationship between the cross-reacting animal species. Thus, although the assay is useful in differentiating between different groups of ruminants, it does not differentiate between closely related animals (e.g., bovine vs. buffalo, sheep vs. goat). The lack of reactivity with chicken and salmon DNA, and DNA from the exempted species pig and horse, indicates that this procedure exhibits suitable specificity to distinguish between banned and exempted animal species in feeds.

The detectability of the assay in the analysis of feeds with actual meat-and-bone meal supplements was not examined in this study. However, the ability of the assay to detect trace amounts of heat-treated meat tissue in a grain-based feed matrix has been

effectively demonstrated using simulated meat meal blends. Furthermore, the assay successfully detected bovine and lamb meals at the 0.1 % (w/w) level in externally acquired feed samples. The detectability of a minor target species is reduced in the presence of a large excess of a different ruminant species; this may be a consequence of the preferential consumption of the universal primer-complementary sequences by the target DNA present in excess during the amplification reaction. This problem might be overcome by designing a multiplex PCR system using multiple species-specific primer pairs in combination with a CHAS bearing a complementary array of capture probes. However, such a system would lose the simplicity of the present approach in which a single universal primer pair is used for the amplification of target sequences present in multiple animal species.

In addition to the ability of the PCR-CHAS to identify specific ruminant species, the method exhibits the additional advantage that it is rapid, simple and the PCR and CHAS can be carried out in one day. The combined universal PCR-CHAS procedure can be used in conjunction with other methods, such as microscopic examination, to maximize the amount of information which can be obtained in the analysis of feed samples, thus contributing to the risk assessment process. Samples could be screened rapidly using the PCR-CHAS method, and those indicating the presence of ruminant material would in turn be examined by microscopy to determine the presence of meat and bone meal, as well as gauge the amount of contamination.

The primary features of the PCR-CHAS that designate it as a good screening tool are low cost, high sample throughput, and target specificity. The average cost to process a sample by the PCR-CHAS is significantly less than microscopy owing to the ability to process multiple samples more rapidly using less expensive equipment. The most sophisticated equipment required for the PCR-CHAS is a thermocycler and hybridization oven. Each can process multiple samples and both items together can be purchased for approximately twenty thousand dollars. In order for a sample to be processed by a microscopist, he/she must have a stereo microscope and compound microscope at their disposal at an average cost of forty thousand dollars for both. One microscopist can process on average, two samples a day whereas an analyst using the PCR-CHAS could easily do ten or more samples and in principal this could be increased significantly. From a quality system point of view PCR and CHAS controls can be introduced to gauge method effectiveness whereas with microscopy, which is judged on observation on a per sample basis, introducing a quality control would be difficult.

The nature of information obtained by the PCR-CHAS, as compared to microscopy, is also a consideration. Microscopy identifies meat meals but cannot differentiate between prohibited and non-prohibited species, making the method ineffective on its own. The PCR-CHAS allows the investigator to quickly determine the presence of a banned species; the disadvantage of the method arises from its inability to differentiate between feed components like blood (non-prohibited) and meat and bone meal (prohibited). For this reason more descriptive information about a sample would be derived from the use of microscopy as a secondary test on samples that are positive with

the PCR-CHAS, in order to determine the presence of blood or meat and bone meal in a given sample. The PCR-CHAS/microscopy combination would allow samples to be tested rapidly and provide maximal information for samples determined to contain ruminant material.

To further enhance the testing capability of the PCR-CHAS other species could be added to the assay. A PCR system amplifying the principal ruminant species as well as equine, porcine and poultry, combined with a CHAS with the appropriate species-specific probes would further enhance the information provided by this assay.

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