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Monoclonal Antibody-Based Sandwich Elisa for the Detection of Ovine Muscle in Cooked Meat

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MONOCLONAL ANTIBODY-BASED SANDWICH ELISA FOR THE DETECTION OF OVINE MUSCLE IN COOKED MEAT

By

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# TABLE OF CONTENTS

List of Tables .......................................................................................................................... vi
List of Figures ........................................................................................................................ vii
Abstract ........................................................................................................................................ viii

1. Introduction .......................................................................................................................... 1

2. Literature Review.................................................................................................................. 5
   2.1 Prevalence of meat species adulteration ................................................................. 5
   2.2 Species adulteration with ovine meat ................................................................. 7
   2.3 Regulations on meat inspection .......................................................................... 8
   2.4 Methods available for detection of ovine in cooked meat ................................ 10
      2.4.1 Near infrared spectroscopy ................................................................... 11
      2.4.2 High performance liquid chromatography ........................................... 12
      2.4.3 DNA-based PCR techniques ............................................................... 13
      2.4.4 Enzyme-linked immunosorbent assay (ELISA) ................................ 16

3. Materials and Methods ...................................................................................................... 21
   3.1 Materials ................................................................................................................... 21
   3.2 Preparation of meat protein extracts ................................................................. 22
      3.2.1 Preparation of pure meat samples ....................................................... 22
      3.2.2 Preparation of laboratory adulterated meat samples ........................ 23
      3.2.3 Preparation of different heat-treated lamb samples ....................... 23
   3.3 Monoclonal antibodies (MAbs) .......................................................................... 24
   3.4 Indirect ELISA ...................................................................................................... 25
   3.5 SDS-PAGE and Western blot ............................................................................. 26
   3.6 Sandwich ELISA ................................................................................................. 26
   3.7 Statistical analysis ................................................................................................. 27

4. Results and Discussion ...................................................................................................... 28
   4.1 Selection of a pair of MAbs for sandwich ELISA ............................................. 28
   4.2 Characterization of MAbs by indirect ELISA ................................................... 29
   4.3 Characterization of MAbs by Western Blot ....................................................... 30
      4.3.1 Characterization of MAb 7F6 by Western Blot ................................... 30
      4.3.2 Characterization of MAb 6F11 by Western Blot ................................... 31
      4.3.3 Comparison of Western Blot with indirect ELISA .............................. 32
   4.4 Species specificity by sandwich ELISA ............................................................... 32
   4.5 Cross-reactivity with non-meat proteins by sandwich ELISA ....................... 35
LIST OF TABLES

Table 1: Species specificity and reactivity of MAbs 7F6 and 6F11 ................... 47
Table 2: Western blot analysis of meat protein extracts with MAbs 7F6 and 6F11 47
Table 3: Optimized experimental conditions for sandwich ELISA..................... 48
Table 4: Intra- and inter-assay coefficient of variation (CV) ............................. 49
LIST OF FIGURES

Figure 1: Schematic of the sandwich ELISA ....................................................... 50
Figure 2: Screening test of MAbs against cooked ovine proteins ....................... 51
Figure 3: Selection of capture antibody by sandwich ELISA ............................. 52
Figure 4: Species specificity of MAb 7F6 by indirect ELISA ............................ 53
Figure 5: Species specificity of MAb 6F11 by indirect ELISA .......................... 54
Figure 6a: Western blot of raw meat samples with MAb 7F6 ............................. 55
Figure 6b: Western blot of cooked meat samples with MAb 7F6 ........................ 56
Figure 6c: Western blot of autoclaved meat samples with MAb 7F6 ................. 57
Figure 7a: Western blot of raw meat samples with MAb 6F11 ........................... 58
Figure 7b: Western blot of cooked meat samples with MAb 6F11 ..................... 59
Figure 7c: Western blot of autoclaved meat samples with MAb 6F11 ............... 60
Figure 8: Species specificity of the sandwich ELISA ......................................... 61
Figure 9: Cross-reactivity with non-meat proteins by sandwich ELISA ............. 62
Figure 10a: Detection limit of lamb-in-pork by sandwich ELISA ...................... 63
Figure 10b: Detection limit of lamb-in-beef by sandwich ELISA ..................... 63
Figure 10c: Detection limit of lamb-in-chicken by sandwich ELISA ............... 63
Figure 11: Effect of heat-treatment of ovine meat on the sandwich ELISA signals 64
Figure 12: Effect of frozen storage of ovine meat on the sandwich ELISA signals 65
ABSTRACT

Meat species adulteration is a worldwide problem, which violates food labeling laws, constitutes economic fraud, and raises ethical, religious and food safety concerns. In the US retail market, sheep is a major substituting species in other ground meats with a higher violation rate in cooked meats than in raw meats. Mixing of different species followed by grinding and/or heat-processing adds to the difficulties of discrimination of meat origin and limits the detectability of many analytical techniques such as electrophoresis and chromatography. Enzyme-linked immunosorbent assay (ELISA) is a useful tool in meat species identification. Although several commercial ELISA kits using polyclonal antibodies (PAbs) are currently available for the qualitative detection of cooked sheep meat, immunoassays using monoclonal antibodies (MAbs) would offer advantages over PAb-based immunoassay for meat speciation. This study aimed to develop a rapid MAb-based sandwich ELISA for the detection of undeclared sheep content in heat-processed meats.

A pair of MAbs was previously produced using hybridoma technology by cell fusion after immunizing mice with soluble myofibril proteins extracted from heat-treated ovine muscle. MAb 7F6 (IgG1) was used as the capture antibody and MAb 6F11 (IgG2a) conjugated to biotin was used as the detection antibody. The sandwich ELISA constructed with these two MAbs displayed strong reactivity to cooked (100°C, 30 min) ovine muscle proteins. There was no observed cross-reactivity to any of the protein extracts from non-ovine meats (beef, pork, horse, deer, and poultry) and some non-flesh proteins (milk, egg albumin, and gelatin) that are commonly used as food additives in processed meat products. Only soy proteins showed a slight cross-reaction. Laboratory-adulterated cooked meat mixtures including sheep-in-pork, sheep-in-beef, and sheep-in-chicken were prepared at various adulteration levels (0% – 10%, wt/wt) in order to evaluate the sensitivity of the assay. The detection limits for cooked sheep muscle spiked in pork, beef, and chicken were 0.5%, 0.5%, and 0.25% (wt/wt), respectively. The average intra- and inter-assay coefficient of variation was 3.4% and 6.0% for lamb-in-pork, 3.5% and 4.7% for lamb-in-beef, and 3.2% and 5.9% for lamb-in-chicken, respectively. This is the first report of a MAb-based sandwich ELISA that has demonstrated utility in the authentication and/or detection of trace amounts of ovine muscle in heat-processed meat products.
1. INTRODUCTION

Meat species adulteration/substitution or mislabeling of meat products has been reported from different countries such as Canada, Australia, and the United Kingdom (Odumeru 2003; Chemistry Center of Western Australia 1999; MAFF 1999). In the US, several surveys also demonstrate the existence of adulterated meat products. Hsieh and others (1995) investigated Florida retail markets in 1994, and found adulteration rates for cooked meat and raw meat of 23% and 16%, respectively. They also found that sheep meat was the most frequent source of substitution compared to pork and poultry for retail ground beef and veal products tested, with 47% of ground beef and 48% of ground veal samples being adulterated with sheep meat. Another survey conducted by the American Dateline Program in 1997 found that 29 out of 100 ground beef samples were adulterated with up to three kinds of non-beef species – pork, lamb or poultry (Christian Resource Centre, Bermuda 2002; Food Safety Net 1999). The prevalence of species adulteration in processed meat products might be due to either unintended contamination as a result of improper handling or processing, or deliberate adulteration for economic gain. The species substitution is more difficult to detect in processed products than in fresh or intact meat cuts, because after grinding, heating, curing, or mixing, the origin of meat species is easy to be concealed in the meat mixture due to the change of meat texture, color, appearance, or even flavor. The reason for mixing expensive sheep meat with other meats could be the transformation of unmarketable wastes from trimmed sheep meat into profitable ground beef or veal products.

Undeclared sheep meat presented in other meat products not only violates food labeling laws, which require the accuracy of ingredients in food products to be labeled, but also imposes ethical and food safety concerns. Some people avoid sheep meat in their diet because they dislike the flavor of sheep, while others may have allergic reactions to ovine meat (Fiocchi and others 1995a, 1995b; Fuentes and others 2005). Another growing food safety concern is associated with scrapie (prion disease occurred in sheep and goat) due to the increasing awareness of bovine spongiform encephalopathy (prion disease occurring in cattle). The epidemic of BSE, along with its oral transmissibility to humans has highlighted the people’s fear of prion diseases (Brugere-Picoux and others 1995; Horiuchi 2005).
Although scrapie has been experimentally transmitted to primates, rodents, and other species, there is no epidemiological evidence to verify that scrapie can be naturally spread to humans (Johnson 2005). In spite of the unproven connection between the human variant Creutzfeldt-Jakob disease (vCJD, human prion disease) and scrapie, unknown health risks frighten the public away from the scrapie meat. This risk is of more concern than other pathogen–associated diseases because most bacteria or viruses can be destroyed through food processing procedures such as heating or irradiation. However, the transmissible agent of the prion diseases – misfolded isoform of prion protein – was found to be resistant to proteases, nuclease, ultraviolet, ionizing radiations, and high temperatures; thus, common food processing or sterilizing methods cannot inactivate the agent (Prusiner 1982). One effective measure against the possibility of contamination of meat products with scrapie is to ensure the accuracy of meat labeling and to distinguish undeclared ovine muscle from complex meat mixtures with a reliable analytical method. Therefore, the ability to detect cooked sheep in heat-processed food products is imperative for regulatory inspection, consumer protection, and food quality and safety.

To date, four kinds of approaches have been applied for identification of heat-processed ovine meat: near-infrared spectroscopy (NIRS), high performance liquid chromatography (HPLC), DNA-based polymerase chain reaction (PCR) techniques, and polyclonal antibody (PAb)-based enzyme-linked immunosorbent assay (ELISA).

A number of NIRS methods have been developed for analyzing raw meat species (Rannou and Downey 1997; McElhinney and others 1999; Downey and others 2000; Ding and others 1999). One of the NIRS methods was designed for detecting cooked (by microwave oven at 900W for 2 min) beef hamburger adulterated with 5-25% mutton with an accuracy up to 92.7% (Ding and Xu 2000). The mutton content in the meat mixture could be quantified by establishing a calibration model; however, the performance of the calibration equations was affected by sample preparation methods such as heat-processing, and the moisture, fat, and food additive content.

An HPLC method was developed for detecting three species of meat adulterants: sheep, horse, and kangaroo in cooked beef products based on the large ratio difference of the histidine dipeptides – anserine and carnosine – present in different skeletal muscles. Together with electrophoretic mobility analysis of myoglobin in sodium dodecylsulphate
gels, the final definitive identification of the adulterant could be acquired (Carnegie and others 1985). With the rapid development and wide application of PCR and ELISA techniques, liquid chromatography methods are seldom used for meat speciation due to the arduous operation and complicated interpretation of chromatographic patterns, especially for the analysis of meat mixtures or heat-processed products.

DNA-based techniques have an advantage in analysis of heat-processed foods because DNA is more heat-resistant than many proteins. DNA hybridization techniques were first adopted for meat species differentiation (Chikuni and others 1990; Ebbehøj and Thomsen 1991a, 1991b). However, these methods are complicated and time-consuming, and thus are gradually replaced by the polymerase chain reaction (PCR) technique, which is relatively simple and fast. Various PCR analytical systems such as, restriction fragment length polymorphism PCR (RFLP/PCR) (Meyer and others 1995; Wolf and others 1999), multiplex PCR (Matsunaga and others 1999; Rodriguez and others 2004), and real-time PCR (RT/PCR) (Lopez-Andreo and others 2005), have been designed for unequivocally detection of animal materials from different biological origins. The successful application of these techniques in the analysis of raw, cooked or even autoclaved products with high sensitivity and specificity demonstrates their powerful ability to detect the target DNA fragment. However, some of the drawbacks associated with any DNA-based procedures include susceptibility to contamination, requirements for expensive equipment or reagents, complexity in quantitative analysis of complex meat mixtures, as well as DNA yields that rely on the source material, extraction methods, or DNA fragmentation after heat-processing at high temperature (Hsieh 2005; Lopez-Andreo and others 2005).

Protein-based ELISA techniques have been considered very useful for meat species identification, because they are simple and useful in providing semi-quantitative results and can offer a suitable sensitivity for regulatory purposes. ELISA techniques can be adapted to different formats such as indirect, competitive or sandwich ELISA. Either PAbs or MAbs can be used in the ELISA systems as the capture molecules to trap target molecules in the sample.

The production of PAbs is simple and cost-effective; therefore, many PAb-based ELISA methods have been developed for detection of either raw or heat-processed meat products (Patterson and others 1984; Martin and others 1988a, 1988b; Berger and others
One reported sandwich ELISA based on polyclonal antiserum could differentiate sheep from other species in cooked meat products (Andrews and others 1992) with a detection limit of 0.13% (vol/vol) or above. Some commercial ELISA kits for qualitative detection of heat-treated ovine protein are also PAb-based. For instance, ELISA-TEK® Cooked Meat Species Kit has detection limit of 1.0% in canned, cooked, or processed foods; BioKits® (Cooked) Species Identification Kits can detect less than 2% cooked sheep.

However, the supply of PAbs depends on the survival of individual animals, thus PAbs suffer from limited production and variable affinity. Moreover, extensive affinity-purification is required for eliminating the cross-reactivity to obtain specific identification (Patterson and others 1984). On the contrary, despite the high cost of initial production, once developed, a MAb can be produced in unlimited amounts from its hybridoma cell line, which offers MAbs many advantages over PAbs (Kohler and Milstein 1975). These advantages include continuous supply, uniform specificity and affinity suitable for standardized procedures, and eventually reduced cost for long-term application. Based on MAbs, several ELISA methods have been developed for identification of different species, including raw chicken meat (Martin and others 1988c, 1991); cooked mammalian meats (Hsieh and others 1998), cooked poultry meats (Sheu and others 1998; Djurdjevic and others 2005), heat-processed porcine muscle proteins (Chen and others 2000, 2002a; Liu and others 2006) and rendered ruminant muscle tissues (Chen and others 2002b, 2004). However, by far, there is no reported MAb-based ELISA that can detect ovine in cooked meat mixture.

Therefore, the objectives of this study were: (1) utilizing heat-processed ovine proteins to immunize mice to elicit MAbs which could specifically recognized cooked ovine muscle proteins; (2) using produced MAbs to develop a sensitive sandwich ELISA that could effectively detect low levels of ovine muscle content in heat-treated meat products.
2. LITERATURE REVIEW

2.1 Prevalence of meat species adulteration

Meat species adulteration/substitution means that meat products contain undeclared meat species; as a result, the meat ingredients are not consistent with the label. According to federal and state regulations, adulterated meat products are prohibited from sale to consumers. Regardless of the rules on meat production, selling mixed meat as pure meat and/or mislabeling meat ingredients has been found not only in domestic retail markets, but also in foreign countries, such as Canada, Australia, the United Kingdom, and Turkey (Odumeru 2003; Chemistry Center of Western Australia 1999; MAFF 1999; Ayaz and others 2006).

For example, Ayaz and others (2006) used the Cooked Meat Species Identification Kit (ELISA-TEK, Gainesville, FL) to examine a total of 100 meat and meat products in Turkey. All raw and heat-processed meat extracts were boiled in a water bath for 15 min before analysis. Eleven out of 28 fermented sausages (39.2%), 5 out of 14 cooked salami (35.7%), 3 out of 11 frankfurters (27.2%), 2 out of 9 raw meats (22.2%) and 1 out of 16 raw ground meats and meat balls (6.2%) contained undeclared species. The main adulterants in meat products labeled as beef were poultry or beef and poultry mixture. In total, 22.0% meat samples were in violation of the Turkish Food Codex. This investigation indicated that meat species substitution occurs more frequently in processed products such as ground, comminuted, cured and cooked meats; the same conclusion was also deduced from other studies (Barai and others 1992; Patterson 1985; Hsieh and others 1996).

One possible reason of high adulteration rate occurred in processed meat products is accidental contamination resulting from improper handling or processing. For instance, if the grinder is not cleaned before other meat is put through, ground meat will contain small amounts of the previous ground meat. Another reason is deliberate adulteration of processed meat products with inexpensive meat for economic gain, because it is more difficult to detect adulterant in cooked or ground meat than in fresh or intact meat. After grinding, heating, and/or curing processes which may cause the change of meat texture, color, appearance, or even flavor, the origin of meat is easily concealed in a meat mixture.
Another survey was conducted in 1994 by Hsieh and others (1995) who collected 902 meat samples from over 500 Florida retail markets. The results showed that 22.9% of cooked ground meats and 15.9% of fresh ground meats were substituted with meat from other undeclared species, whereas all intact cuts were accurately labeled. This survey also revealed that sheep meat was the most frequent source of substitution for ground beef and veal compared to pork and poultry. A similar result was obtained from research by Carnegie and others (1985) who employed an HPLC method to monitor cooked beef products. One brand product, “beefsteak” pie, was found to be a mixture of mutton and beef.

A consumer investigation was launched by the “Dateline” television program from August 1997 through November 1997 to examine the authenticity of ground beef. A total of 100 ground beef samples were purchased randomly from 100 supermarkets in 10 cities around the country with 10 stores in each city. All the samples were tested for pork, lamb and poultry by ELISA Technologies Inc. (Gainesville, FL). Twenty-nine out of 100 ground beef samples labeled as pure beef were adulterated with one, two, or even three kinds of non-beef species — pork, lamb or poultry. After this finding, Dateline conducted a follow up survey the next year to find whether the problem of meat species adulteration had improved. Dateline staffers went to 100 supermarkets to collect the samples from 10 cities including the 29 stores that failed Dateline’s ground beef test in 1997. In re-testing, adulterated meat was not found in any of the 29 stores which previously failed the compliance. However, adulterated meat samples were found in some of the stores that passed Dateline’s test in 1997. They mixed pork or lamb in ground beef (Christian Resource Centre, Bermuda 2002; Food safety net 1999). Results from this investigation demonstrate that economic fraud can not be prevented without effective surveillance.

It is a common belief that species substitution only occurs by mixing cheap meat in expensive meat. In the US retail market, pork and poultry meats are usually less expensive than beef and lamb meats, thus, beef products are supposed to be adulterated with poultry or pork meats. The reason for using lamb meat as adulterant is actually an action of “changing waste to profit”, by utilizing unmarketable lower-value sheep trimmings to increase the bulk of high-grade ground meats (such as beef or veal) for economic gain (Hsieh and others, 1995).
2.2 *Species adulteration with ovine meat*

According to the state and federal laws, meat products must be labeled as to their contents. Grinders are required to be completely disassembled and sanitized between grinding different meat species to prevent contamination of the meat that remained in the grinder from previous grinding process. Therefore, no matter how much the adulterant content is, if the meat contains undeclared species, it is deemed illegal and misbranding.

Muscle food products adulterated with sheep meat is intolerable to people who dislike the flavor of mutton or lamb. Besides, Some cultural group, such as semi-vegetarians, choose not to eat certain types of meat (commonly "red meat" including lamb) while still consuming poultry and fish for health or ethical reasons (Donovan and Gibson 1996). They believe, for example, that it is inhumane to slaughter large animals. In this case, poultry products containing ovine ingredient is unacceptable for them.

Undeclared ovine meat as adulterant in other meat products may also pose an allergic risk for sensitive individuals. Several reports indicated that ovine meat could cause allergic reaction in both sensitive children and adults (Fiocchi and others 1995a, 1995b; Fuentes and others 2005). Sometimes, even anaphylaxis, a severe allergic symptom, could be triggered after the ingestion of lamb meat (Welt and others 2005). In one case, a cat had a food hypersensitivity to lamb, and severe facial pruritus was caused by an exclusive diet of lamb (Reedy 1994).

The most important concern for consumers, scientists and governments maybe the species-related disease which can be transmitted to human. A non-bacterial disease known as scrapie is raising concern among people, because the occurrence and spread of bovine spongiform encephalopathy (BSE) is thought to be established by feeding cattle with scrapie-infected sheep tissues (Wilesmith and others 1988).

Scrapie, which occurs in sheep and goat, is the prototype of the transmissible spongiform encephalopathies (TSE) and has been recognized for more than 250 years. TSE is also known as prion diseases because the pathogenesis of TSE is the conversion of normal host prion protein (PrPc), into an abnormal misfolded isoform, protease-resistant PrP (PrPres) or scrapie PrP (PrPsc). Bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of wild ruminants, transmissible mink encephalopathy (TME),
Creutzfeldt-Jakob disease (CJD) and its emerging new variant (vCJD) in humans all belong to the group of TSE, because they share the same characteristics: a prolonged incubation period, fatal neurodegenerative disorders, spongiform changes in the central nervous system, and transmission within and between species by ingestion or inoculation (Brugere-Picoux and others 1995; Caramelli and others 2006, Chesebro and others 2005). Pathogenetic studies demonstrated that PrP(sc) is concentrated in the enteric nervous system and in the gut-associated lymphoid tissue following an oral scrapie ingestion (Caramelli M and others 2006). However, several experiments on mouse and hamster models showed that skeletal muscles were capable of accumulating substantial titers of prions after oral consumption of scrapie. (Bosque and others 2002; Thomzig and others 2003). These findings suggested that the muscles of infected animals could also contain infective TSE agents.

It has been well-documented that prion diseases can be orally transmitted to humans who consume infected cattle materials. Scrapie is endemic in flocks in many countries. Presently, there is no epidemiological evidence to verify that scrapie can be transmitted to human. The possibility of human infection from scrapie is of great concern but is still unidentified. In spite of the negative linkage between human CJD and scrapie, unknown health risks frighten the public away from meat products contaminated with scrapie-infected mutton or lamb meat. Besides, it was found that the transmissible abnormal prion protein could not be inactivated by proteases, nuclease, ultraviolet, ionizing radiations, and high temperature (Prusiner 1982); the TSE agents could survive in cooked, canned or autoclaved food products. Therefore, avoiding meat products containing undeclared species is an effectual way to guarantee the consumption of wholesome food.

In summary, an effective and reliable analytical method for the detection of ovine muscle tissue mixed in cooked meat products would be significant in: (i) discouraging the violation of Federal food labeling laws to prevent economic fraud and protect consumer’s benefits; (ii) avoiding ethical issues regarding cultural beliefs or humane reasons; and (iii) ensuring the quality and safety of muscle food to reduce the health risk resulted from allergy to undeclared sheep ingredients, or species-related pathogens.

2.3 Regulations on meat inspection
Regulations regarding meat safety and quality play an important role in developing new technologies and improving existing techniques which are used for meat inspection and analysis, thereby ensuring that the products meet the regulatory requirements.

The US (federal) meat inspection program began on August 30, 1890, when Congress passed the initial Meat Inspection Act for salted pork and bacon for export. In 1891, the Act was amended to cover the inspection and certification of all cattle as well as the beef meat for export, and then expanded again in 1894 to include pork (FDA 2002). In 1906, Congress responded to the public requests for improved working environments and better sanitary conditions by passing the Federal Meat Inspection Act (FMIA), which provided the inspection of cattle, hogs, sheep, horses, goats as well as their carcasses destined for interstate and foreign commerce. In addition, the inspection also covered processed meat, meat equipment and facilities to prevent the contaminated meat products from being sold to domestic consumers (FDA 1906). After World War II, there was a rapidly expanding market for poultry products. In response to the rapid growth of poultry industry, Congress approved the Poultry Products Inspection Act (PPIA) in 1957 to extend U.S. Department of Agriculture’s (USDA) Food Safety and Inspection Service's (FSIS) jurisdiction to the inspection of poultry products (FDA 1957).

To strengthen the safety of meat supply, the Wholesome Meat Act of 1967 and the Wholesome Poultry Products Act of 1968 updated the 1906 and 1957 Act to include mandatory inspection of all meat processed and sold within the same state (intrastate inspection). The 1967 and 1968 Acts also addressed mislabeling issues by describing under what terms a carcass or poultry product may be classified as "adulterated". Thus, all meat products intended for human consumption must be inspected for safety, wholesomeness, and accurate labeling including both ingredient labeling and nutritional labeling, when applicable. Under the Meat Act the USDA/FSIS inspectors are also required to enforce the meat products uniformity standards, so the product remains consistent across different processing plants (FDA, Food Code 2001; FDA 1968).

In the US, meat production is the most highly regulated food industry. The USDA’s FSIS is responsible for developing rules and regulations such as labeling guidance for safe and wholesome food, administering programs, as well as providing regulatory oversight.
On January 6, 1993, the U.S. Food and Drug Administration (FDA) published final regulations amending the Nutrition Labeling and Education Act of 1990, which requires nutrition labeling of most foods regulated by the FDA. The amended final regulations require the listing of the common or usual names of all ingredients in standardized foods, as well as comparable nutrition labeling for multi-ingredient and heat processed meat and poultry products (FDA 1993). FSIS is the agency responsible for ensuring the truthfulness and accuracy in labeling of meat and poultry products to protect consumers from misbranded and economically adulterated products. On the other hand, FDA regulates all other food labeling, including food ingredients.


\textbf{2.4 Methods available for detection of ovine in meat products}

A variety of analytical approaches have been developed for meat species identification. However, two main problems hinder the application of most of these methods for the detection of ovine meat in cooked food products. First, many analytical approaches are only valid for the analysis of raw meat materials but not heat-processed samples. Part of the reason is that these techniques are based on thermal-labile proteins. When the target protein is denatured after heating, the detection ability decreases or even disappears. For instance, blood serum protein based immunoassays have less reliability in detecting cooked meat due to the heat-susceptibility of serum proteins (Dincer and others 1987). Second, certain types of analytical techniques are incapable of detecting meat origin in complex meat mixtures. These techniques, such as electrophoresis and chromatography, may be successful in differentiating individual meat species, but they are less sensitive and reproducible to detect a specific meat species in meat mixtures due to the difficulty in interpreting the data obtained from complex protein bands or chromatographic patterns.
For example, sodium dodecyl sulfate polymer-filled capillary gel electrophoresis (CE-SDS) is a sensitive protein analytical method which can separate hundreds of components at the same time and is easily automated (Cota-Rivas and Vallejo-Cordoba 1997). Vallejo-Cordoba and Cota-Rivas (1998) utilized CE-SDS to analyze raw beef, pork and turkey meats. The accurate classification with linear discriminant analysis (LDA) of water-soluble protein was 100% for beef and turkey, 94% for pork. A classification of saline-soluble protein was 88% accurate for beef and mechanically deboned turkey meat, and 94% and 100% for turkey and pork meat, respectively. Although this method was effective for distinguishing each meat species, it was difficult to analyze the complicated protein profiles if meat product contained multiple species or protein additives. Besides, it was invalid to detect heat-treated meats.

Therefore, only the following four kinds of techniques have the capability and have been applied for analyzing heat-processed ovine in mixed meat products: near infrared spectroscopy (NIRS), high performance liquid chromatography (HPLC), DNA-based PCR techniques, and Enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies. Each method has its advantages and is companied by some limitations.

2.4.1 Near infrared spectroscopy

The principle of NIRS is that the absorption of the electromagnetic spectrum occurs in the near infrared region, which is defined as the wavelength range of 780–2526 nm by the American Society of Testing and Materials (ASTM). The absorption bands are produced by specific molecules in the analyzed samples, and are related to overtones and combinations of the fundamental vibrations (Ciurczak 2001). NIRS has been employed for determination of chemical composition in poultry breast or thigh meat (Berzaghi and others 2005; Cozzolino and others 1996), for differentiation of fresh and frozen beef (Downey and Beauchêne 1997a, 1997b; Thyholt and Isaksson, 1997), and for discrimination of meat species (Rannou and Downey 1997; McElhinney and others 1999; Downey and others 2000; Ding and others 1999).

Most NIRS methods designed for species identification were limited to analyzing raw minced meat samples. For instance, McElhinney et al. (1999) utilized visible, near and mid-infrared spectroscopy for quantitation of lamb content in raw minced mixtures (5%, 10%
and 20% lamb-in-beef, wt/wt). Only one study examined the application of NIRS for detecting cooked mutton. In this study, cooked beef hamburgers (by microwave oven at 900W for 2 min) were partially substituted with 5-25% mutton, pork, skim milk powder, or wheat flour, respectively (Ding and Xu 2000). Compared to raw mixture and cooked hamburgers, minced cooked hamburgers gave the best discriminating performance with classification accuracy up to 92.7% by KNN (K-nearest-neighbor) method. The accuracy was increased with the increase of the adulteration level. For example, the percentages of misclassification for minced cooked hamburgers at 5%, 15% and 25% adulteration level were 22.2%, 5.6% and 2.8%, respectively. Once adulterant such as mutton was detected, its content in meat mixture could be further quantified by establishing a calibration equation.

NIRS is rapid, non-destructive, and capable of both qualitative and quantitative analysis. However, the performance of the calibration equations can be influenced by cooking method, fat content, moisture level, or different food additives in analytical samples (Ding and Xu 2000). In addition, large database for reference to develop a calibration model, professional knowledge for data processing, and expensive equipment are required (Scotter 1997; Gizzi and others 2003). Besides, the sensitivity and accuracy still needs to be improved for obtaining reliable results. These disadvantages obstruct NIRS technique from becoming a routine analysis of meat origin.

2.4.2 High performance liquid chromatography

Several liquid chromatography (LC) methods have been reported for identification of meat species. An early application of LC technique for detection of chicken meat was based on the significant ratio difference of the histidine dipeptides – anserine/carnosine (a/c) – presenting in different skeletal muscles among pork, beef and chicken. The high a/c ratio for chicken meat could enable the detection of 5% chicken in both cooked pork and pork-beef mixtures (Tinbergen and Slump 1976). Carnegie and others (1985) also utilized the big difference of a/c ratio to develop an HPLC method for the detection of sheep, horse, and kangaroo meats in cooked beef products. The final definitive identification of the adulterant could be acquired by combining the electrophoretic mobility analysis of myoglobin in sodium dodecylsulphate gels. Using this method, the authors found that one beef product was actually a mixture of mutton and beef. On the basis of pork fat having
large amounts of triglyceride which contains saturated fatty acid at the C-2 position, Saeed and others (1989) developed a LC method to distinguish pork meat and lard from other species, and addition of 1% pork-in-beef and 3% pork-in-mutton could be detected in both raw and heat-processed meat. This assay utilized fat independent of heat treatment to expand its application into cooked meat products, but the content of fat in pork varied broadly, and this inherent drawback made the quantitative analysis impossible (Hsieh 2005).

HPLC technique has been seldom used for meat speciation in recent years because of the complex nature of the chromatographic pattern when mixed species or heat-processed products were analyzed. Expensive equipment as well as laborious sample preparation also discourages the application of this method.

### 2.4.3 DNA-based PCR techniques

DNA-based techniques have attracted considerable attention during the past decade and have been successfully applied in species identification. The advantages of DNA analysis have been reviewed as follows (Lockley and Bardsley 2000): (1) DNA is more stable and less liable to high temperature, so DNA-based techniques are suitable for analysis of heat-processed products; (2) the presence and characteristics of DNA are independent of the cell types, i.e. identical genetic information is contained in different samples such as blood, muscle or bone; (3) DNA has abundant information content due to the degeneracy of the genetic code. For these reasons, DNA-based methods are widely used in the nutrition and food area.

DNA hybridization and DNA-based polymerase chain reaction (PCR) techniques are two major methods for meat speciation. The former approach has been obsolete because it is complex and time-consuming. On the contrary, PCR techniques have been developed quickly in recent years for their relative simplicity and speed. A number of PCR-based methods have been designed for meat species identification. Random amplified polymorphic DNA (RAPD), multiplex PCR, restriction fragment length polymorphism PCR (RFLP/PCR), and real-time PCR (RT/PCR) are common approaches used currently. Several articles (Lopez-Andreo and others 2005; Sun and others 2003; Hsieh 2005) provide comprehensive reports on using different PCR methods for the detection of animal species.
Also, the classification of DNA-based assays, as well as the advantages and limitations for each category, were summarized by Hsieh (2005).

An early study (Chikuni and others 1994) based on oligonucleotide primers for the amplification of sheep satellite I DNA sequence could qualitatively discriminate between sheep, goat and bovine meats. A detection limit of 10 pg of DNA from sheep meat could be achieved by 4% agarose gel electrophoresis after PCR amplification. Heat treatment caused reduced PCR products, but sheep DNA still could be detected in the meat mixtures after being heated to 120°C using this method. Further differentiation of sheep from goat was obtained by Apal digestion of amplified PCR products because sheep had one Apal site in the PCR products while goat had none.

By using a conserved DNA sequence in the mitochondrial (mt) cytochrome (cyt) b gene to design a forward common primer, and species-specific DNA sequences for reverse primers, Matsunaga and others (1999) developed a multiplex PCR to identify six meats including sheep, goat, beef, pig, horse, and chicken. Sheep and goat fragments could be detected after heating at 100°C or 121°C for 30 min, and this assay had a detection limit of 0.25 ng for all meat DNA samples. In another multiplex PCR, the forward primer was designed on mt 12S ribosomal RNA gene (12S rRNA). This assay could specifically and qualitatively detect pork, beef, sheep, and goat in raw, pasteurized (65°C for 30 min) and sterilized (121°C for 20 min) meat mixtures. The detection limit was 1% (wt/wt) for sheep or goat meat mixed in pork (Rodriguez and others 2004).

As an alternative to direct DNA sequencing after PCR amplification, restriction fragment length polymorphism (RFLP) PCR utilizes restriction enzymes digesting amplified DNA fragments to detect genetic variation among species. On the basis of amplification of mt cyt b gene by PCR-RFLP, Meyer and others (1995) employed 20 different endonucleases to discriminate more than 22 animal species including sheep, goat and other game meats in marinated and heat-treated products. Wolf and others (1999) also selected a specific region on mt cyt b gene for amplification using PCR-RFLP method. They investigated 25 game species using 11 restriction enzymes (REs) and found that two different REs were sufficient to differentiate all the game meats including sheep and goat. The effect of heat-treatment to the meat products on the assay performance was not examined in this study.
A fluorescent PCR-RFLP method was designed to amplify partial sequences within the mt 12S rRNA gene followed by fluorescence sensor capillary electrophoresis (Sun and Lin 2003). Species identification was conducted by testing binary mixtures of the cooked (100ºC for 30 min) and autoclaved (121ºC for 30 min) pork, goat and beef at levels of 1, 2, 5, 10, and 50% (wt/wt). Based on the presence of 351 and 167 bp DdeI fragments, goat could be detected in mixtures with pork or beef at 1% level.

Conventional PCR techniques usually require secondary assay, such as DNA sequencing or RFLP to increase the capability of meat species identification. Real-time PCR is an improved technique which utilizes TagMan probes labeled with a fluorescent dye and a quencher to monitor the PCR amplification. Because the fluorescent dye can be released from the quencher at each PCR cycle when the probe matches the sequence of the PCR amplicon, the emission of fluorescence can be detected either by a real-time PCR instrument or a fluorescence spectrophotometer without requirement of further electrophoresis assay. Therefore, this approach can shorten the reaction time, reduce the carry-over contamination, and increase the sensitivity (Hsieh 2005; Lopez-Andreo and others 2005). A real-time PCR system based on six TagMan probes was designed for identification and quantitation of six species: bovine, porcine, ovine, chicken, turkey, and ostrich DNA in complex sample mixtures, which contained two to four species DNA (vol/vol). This assay could detect more than 5% of cattle or lamb, however, the quantitation accuracy reduced as the samples contained less than 10% target DNA (Lopez-Andreo and others 2005).

In general, DNA-based PCR techniques are considered to be highly sensitive, species-specific, and applicable to heat-processed products. Unfortunately, nucleic acid based methods have several drawbacks when applied to the routine analysis. First, the “high sensitivity” of the PCR can also mean that even very small amounts of contamination with the target DNA will result in a false-positive signal, and this is the major limitation of PCR method. Hence, confined and dedicated facilities and additional precautions are required to prevent the cross-contamination (Gizzi and others 2003). Second, the applications of PCR are limited by relatively high cost derived from expensive equipment and reagents, especially for real-time PCR in which fluorescently labeled specific primers and a real-time thermocycler is required (Lopez-Andreo and others 2005; Gizzi and others 2003).
the quantification of species is highly complicated as low levels of adulterant in food matrix, and the data interpretation is quite difficult when dealing with complex meat mixtures. Forth, the yields of DNA can be affected by different source materials, extraction method, or DNA fragmentation created from processed food, therefore, it is difficult to compare quantitative results when analyzing species in DNA mixtures independently due to the lack of a reference standard (Hsieh 2005; Lopez-Andreo 2005). These limitations hinder the routine use of DNA-based PCR techniques for the food authenticity control.

2.4.4 Enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies

Enzyme-Linked Immunosorbent Assay (ELISA) methods are immunoassay techniques used for detection or quantification of a substance on the basis of specific antigen-antibody reaction (Kemeny 1991). ELISA, as a fundamental and powerful tool, is already widely used in many fields such as medicine, pharmaceutics, environmental sciences, and agriculture. Food scientists have successfully applied ELISA methods in meat speciation for more than two decades (Whittaker and others 1982). Because ELISA techniques are rapid, sensitive, low in cost, easy to use, and readily automated for high throughput and quantification of analyte concentrations, they are more practical for routine use than other techniques.

ELISA methods rely on either polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs) that capture the target antigen in a sample matrix; the success of the analytical system requires strong affinity and high specificity of the antibody (Ab) towards the target antigen (Ag). PAbs are produced by immunizing animals with immunogen to obtain antiserum, which often suffer from cross-reactivity to closely related species; thus requiring laborious procedures for eliminating the cross-reactive components (Patterson and Jones 1990). MAbs overcome the major shortcomings that are associated with PAbs, because the production of MAbs is based on cell fusion technique. By fusion to a myeloma cell, an individual B cell can be converted to a hybridoma cell line to ensure an unlimited production of a homogenous antibody reagent (Kohler and Milstein 1975). MAbs produced by a single B-cell clonal line have an inherent mono-specificity toward a single epitope (also called antigenic determinant) that allows fine detection and quantitation of small
differences in antigen. Epitope is the part of an antigen that can directly interact with antigen receptor molecules (antibodies).

ELISA systems can be adapted to different formats such as indirect, sandwich, and competitive ELISA. In contrast to indirect ELISA, which involves only one antibody specifically interacting with antigen, sandwich ELISA requires double antibodies (capture Ab and detection Ab) capable of recognizing two non-overlapping epitopes on the same antigen molecule. The binding of the capture Ab to antigen cannot obscure or change the epitope recognized by the detection Ab. The stringent prerequisite for constructing a successful sandwich ELISA offers this immunoassay format a big advantage over indirect ELISA: i.e. sandwich ELISA is particularly powerful in analyzing small amounts of target molecules, even though they exist in contaminated food matrices. Because the sensitivity and specificity of the sandwich assay are enhanced as a result of the target molecules being recognized by the double Abs simultaneously (Palomäki 1991). Correspondingly, it is not necessary to centrifuge or filter the sample to get purified antigen, so the sample preparation is simple and rapid; thus, sandwich ELISA is suitable for analyzing large amount of samples, and can be used as a routine tool for large-scale screening tests. On the other hand, if a purified antigen standard is prepared, the sandwich assay can determine the absolute amount of antigen in an unknown sample, and accurate quantitative result can be obtained. Competitive ELISA can be either direct or indirect, and involves a plate-bound target molecule in competition with sample analyte for available capture molecule-binding sites (Brocchi and others 1990).

A number of ELISA techniques use PAbs for the identification of species origin in either raw meat mixtures (Patterson and others 1984; Martin and others 1988a, 1988b) or heat-processed products (Berger and others 1988, Kang’Ethe and Gathuma 1987; Andrews and others 1992; Ansfield and others 2000a, 2000b). Several MAbs with specificity to poultry (Martin and others 1988c; Billett and others 1996), equine (García and others 1994), and pork (Morales and others 1994) have been developed for meat speciation, but only limited to raw meat samples. Comprehensive and detailed information about these methods was provided in several review articles (Hitchcock and Crimes 1985; Patterson and Jones 1990; Hsieh 1999, 2005).
More recently, a series of MAbs against soluble heat-resistant muscle proteins were produced for determination of species content in heat-processed products. Chen and others (2002a, 2002b) demonstrated that a marker protein, skeletal troponin I (sTnI), could be easily extracted from heat-treated animal skeletal muscles with salt or phosphate buffered saline solution. MAbs against this soluble thermal-stable sTnI could be produced by immunizing mice with either crude protein extracts from heated skeletal muscles or pure sTnI. Several ELISA systems based on different species-specific MAbs were developed, and each of them displayed corresponding specificities towards meat species.

Indirect ELISA based on MAb 2F8, against cooked pork protein extract, could identify cooked mammalian meat (beef, sheep, horse, pork and deer) with a detection limit of 0.5% by weight (Hsieh and others 1998). Poultry specific MAb 5D2 was characterized and utilized for developing both indirect competitive and non-competitive ELISAs, which could quantitatively detect cooked chicken or turkey in mammalian meats. As low as 1% detection limit could be achieved with the overall accuracy ranging from 95% to 98% (Sheu and others 1998; Djurdjevic and others 2005). An indirect ELISA based on MAb 5H9 which was specific to porcine sTnI could detect 0.5% (wt/wt) pork in cooked beef mixtures (Chen and Hsieh 2000). Among a group of MAbs against bovine sTnI, MAbs 1F9, 2G3, and 7F7 showed reaction to the TnI of all animal species including mammalian, poultry, and fish; MAbs 7A12 and 8A12 could recognize mammalian TnI, while MAb 2A8 was specific to ruminant TnI (Chen and others 2002b). Three indirect ELISAs based on MAbs 7F7, 7A12 and 2A8 could correspondingly identify rendered (132°C/2 bar for 2 hours) animal muscles, mammalian, and ruminant muscles adulterated in the feedstuffs with 1% to 50% levels. According to the absorbance readings, the detection limits for all species assay was less than 1%; for mammalian and ruminant assays were estimated from 0.3 – 2% (wt/wt).

To improve the detectability, a ruminant sandwich ELISA based on a pair of MAbs 5G9 and 2G3 was further developed that could quantitatively determine 0.05%, 0.5% and 5% (wt/wt) bovine or ovine meat meal (132°C/2 bar for 2h) adulterated in feed samples. The detection limits for bovine and ovine TnI were 5.0 and 4.0 ng/ml, respectively (Chen and others 2004). The porcine indirect ELISA (Chen and Hsieh 2000) was also improved by adapting the sandwich format. MAb 8F10, specific to mammalian sTnI, matched porcine
sTnI specific MAb 5H9. Sandwich ELISA using 8F10 as the capture Ab and 5H9-biotin as the detection Ab could effectively detect porcine proteins from meat mixture. The detection limits could reach 0.05% (wt/wt) for pork-in-chicken, and 0.1% (wt/wt) for pork-in-beef. Whereas, the indirect assay based on MAb 5H9 had only 0.5% (wt/wt) detection limit for pork-in-beef samples. The improved porcine sandwich assay was used to test raw, cooked, autoclaved meat extracts, as well as commercial rendered porcine meat and bone meal (MBM) sample, results indicated that MAbs against sTnI were highly heat-resistant (Liu and others 2006).

Although MAb-based ruminant and mammalian assays appear able to recognize heat-treated ovine muscle, they are not mono-species specific. Individual ovine species can not be distinguished from other ruminant species such as beef and deer, or other mammalian meats including pork, beef, deer, and horse. Presently, all of the ELISA methods designed for detection of single cooked sheep rely on PAbs.

One reported sandwich ELISA based on antiserum could identify beef, sheep, horse and deer in cooked meat products. In tests of diluted cooked extract mixtures, the detection limit of the assay could reach 0.13% (vol/vol) or higher (Andrews and others 1992).

Several commercial ELISA kits currently available for qualitative detection of cooked sheep also utilize PAbs. For example, ELISA-TEK™ Cooked Meat Species Kits (ELISA Technologies, Gainesville, FL) can qualitatively detect 1% and above sheep in cooked, canned or processed foods; BioKits (Cooked) Species Identification Kits (Tepnel BioSystems Limited, U.K.) offer qualitative tests which can detect < 2% sheep in cooked or even autoclaved meat samples.

As mentioned before, MAbs are not subject to variability or exhaustion of stocks as are polyclonal antisera. MAbs possess consistent and stable specificity, unlimited and continuous production, as well as low cost for long-term commercial application and large-scale routine analysis (Patterson and others 1984; Hsieh 1999). Therefore, immunoassays for the detection of cooked ovine can greatly benefit from the use of MAbs, and ovine sandwich ELISA based on MAbs is in great demand.

This research will greatly assist food regulatory bodies and food industries by development of a MAb-based sandwich ELISA with significant advantages in speed, sensitivity and simplicity to identify cooked ovine muscle proteins in complex food
ingredient mixtures, thereby ensuring meat products as being high quality and authentic, and preventing the occurrence of meat species adulteration by either accidental or intentional practice.
3. MATERIALS AND METHODS

3.1 Materials

Chemicals and reagents. All chemicals and reagents used in this study were analytical grade. Sodium chloride, sodium phosphate, citric acid, sodium bicarbonate, sodium carbonate, Tween-20, gelatin, bovine serum albumin (BSA), egg albumin, and N,N-Dimethyl Formamide (DMF) were purchased from Fisher Scientific (Fair Lawn, NJ). Hydrogen peroxide (30%), biotinamidocaproate N-hydroxysuccinimide (NHS-CA-biotin), 2,2'-azino-di-3-ethyl-benothiazoline-6-sulfonic acid (ABTS), horseradish peroxidase (HRP) conjugated goat anti-mouse IgG, streptoavidin-HRP conjugate and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO).

The equipment, buffers, and reagents used for performing SDS-PAGE and Western blot were purchased from Bio-Rad Laboratories (Hercules, CA). Mini-Protean III Electrophoresis Cell, 30% Acrylamide/ Bis Solution, 37.5:1 (2.6% C), 1.5 M Tris-HCl buffer (pH 8.8), 0.5 M Tris-HCl buffer (pH 6.8), 10% (wt/vol) Sodium dodecyl sulfate (SDS) solution, ammonium persulfate (APS), Tetra-methyl-ethylenediamine (TEMED), and 10X Tris/Glycine/SDS Buffer (Running Buffer, pH 8.3) were used for SDS-PAGE. Mini Trans-Blot Electrophoretic Transfer Cell, Precision Plus Protein Kaleidoscope Standards (Catalog No. 161-0375), Nitrocellulose membrane, 10X Tris/Glycine Buffer (Transfer buffer), 10x Tris Buffered Saline (TBS), 0.05% (vol/vol) Tween-20 in TBS (Washing Buffer, TBST), 1% (wt/vol) BSA in TBS (Blocking Buffer), 1% (wt/vol) BSA in TBST (Antibody Buffer), Conjugate antibody – Goat Anti-Mouse IgG (H+L)-Alkaline Phosphatase (AP) Conjugate, as well as Alkaline Phosphatase chromogen kit, i.e. 5-bromo,4-chloro,3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT), were used for Western blot.

Glassware was washed using Versa-Clean® detergent (Fisher Scientific) and rinsed with pure water from the NANOpure DIamond® ultrapure water system (Barnstead International, Dubuque, IA). All the solutions used in this study was prepared using pure water.
Meat samples. Meats from eight food animal species were used to formulate laboratory samples. Fresh pork ham, beef round, lamb leg, whole chicken, turkey breast and turkey thigh meats were purchased from local supermarkets. Horse meat was obtained from the college of Veterinary Medicine, Auburn University (Auburn, AL). Deer meat was supplied by the Fats and Proteins Research Foundation (Bloomington, IL). Each kind of lean meat sample was prepared by trimming off the connective tissue and visible fat, grinding twice, mixing thoroughly, and then was divided into two parts. One part was used to prepare the first batch (batch #1) of samples immediately after grinding, this batch included raw, cooked and autoclaved pure meat protein extracts (totally eight species), as well as laboratory adulterated cooked meat mixture samples (three types including lamb-in-pork, lamb-in-beef, and lamb-in-chicken). Another part was stored in the freezer at –80°C, and ground lamb was thawed six months later for the preparation of the second batch (batch #2) of samples, which were heated in six different conditions for testing the effect of heat-treatment on the assay efficiency. Meats from different species were processed separately. The grinder and utensils were cleaned carefully between samples to prevent cross contamination.

3.2 Preparation of meat protein extracts

3.2.1 Preparation of pure meat samples

The first batch of meat samples were immediately extracted after grinding without freezing and thawing procedure. Ten grams of each kind of ground meat was mixed (1:5 wt/vol) with 50 ml of 0.01 M phosphate buffer saline containing 0.5 M sodium chloride (PBS-NaCl, pH 7.2) for raw meat sample extraction, followed by blending the mixture in a stomacher (Lab-Blender 400, Tekmar Co., Cincinnati, Ohio, U.S.A.) for 1 minute. To prepare cooked meat samples, 20 g of ground meat in a beaker covered with aluminum foil was heated in a boiling water bath for 30 minutes. After cooling, 2-fold (wt/vol) volume of PBS-NaCl was added to each sample. Then the cooked mixtures were homogenized with a homogenizer (T25 Basic S1, IKA Works Inc., Wilmington, NC) at 11,000 rpm/min for 2 minutes. Another set of meat samples was placed in the beakers covered with aluminum foil and then autoclaved in a Benchtop autoclave (NAPCO 8000-DSE, Jouan, Inc.,
Winchester, VA) at 121°C/1.2 bar for 30 minutes. The subsequent procedures were the same as the cooked samples. All the raw, cooked and autoclaved sample homogenates were held at 4°C for 2 hours and then centrifuged (3,220 x g) at 4°C for 30 minutes. The supernatants were filtered through Whatman No. 1 filter paper (125mm dia, Fisher Scientific), aliquoted into small centrifuge tubes (1.5 ml) and stored at -20°C until use. The protein concentration of each of the meat extracts was determined using Protein Assay Kit II (Catalog No. 500-0006, Bio-Rad) with standard procedures for Microtiter Plates Assay according to the manufacturer’s instructions (BSA was used as the protein standard).

Some non-flesh proteins including salmon, ovine blood, bovine serum albumin (BSA), soy proteins, egg albumin, milk proteins and gelatin, were prepared to examine the cross-reactivity of the ovine assay. Egg albumin, BSA, soy protein concentrate, non-fat dry milk, and gelatin were dissolved at 10% (10 g/100 ml) level in deionized water and mixed well. Then the five kinds of mixtures along with minced salmon and sheep blood were cooked in water bath at 100°C for 30 min, after cooling, each sample was homogenized and extracted with PBS-NaCl at a ratio of 1:2 (10g: 20ml). Therefore, the final content for each of the proteins was equal to 10g/120 ml. Whereas, for cooked meat extracts, the content was 10g/20 ml. The following centrifugation and filter procedures were the same as described for the pure meat protein extraction.

3.2.2 Preparation of laboratory adulterated meat mixture samples

In order to evaluate the sensitivity of the sandwich assay, fresh ground lamb meat was mixed with ground pork and ground beef, respectively, at six adulteration levels: 0%, 0.5%, 1%, 2%, 4%, and 10% (wt/wt); and also mixed with ground chicken meat at seven levels with additional level of 0.25%. Each of these samples was thoroughly mixed, and then cooked in a water bath at 100°C for 30 min. The subsequent protein extraction followed the procedures of pure meat extraction as described above.

The adulterated meat samples and pure meat samples were all freshly prepared immediately after purchasing without freezing storage in the freezer.

3.2.3 Preparation of different heat-treated lamb samples
To examine the effect of cooking and autoclaving duration on the performance of the sandwich assay, the second batch of samples (total six) were prepared using ground lamb meat which had been stored in freezer at – 80°C for six months after purchasing and grinding. The ground lamb meat was thawed to about 4°C, and then well mixed and divided into six equal parts, each part was treated with different heating conditions: cooking (100°C water bath) for 15, 30, and 60 min, and autoclaving (121°C/1.2 bar) for 15, 30 and 60 min. The subsequent protein extraction procedures were the same as the first batch of meat samples.

Among the six of batch #2 samples, two of them were treated in the same manner as the counterparts in batch #1, i.e. cooked lamb for 30 min and autoclaved lamb for 30 min. The only difference was that the batch #1 samples were prepared with fresh ground lamb meat immediately after purchasing without freezing and thawing procedures; whereas, the batch #2 samples were extracted using the ground lamb which had been stored at – 80°C for 6 months. Therefore, these four kinds of lamb meat samples (two cooked and two autoclaved lamb extracts prepared at different time) were used to examine the effect of freezing storage on the assay efficiency. The history of the storage of the lamb meat was unknown before purchasing. Therefore, the six-month frozen storage was just relative to the freshly purchased lamb meat.

3.3 Monoclonal antibodies (MAbs)

MAbs were produced using hybridoma technology by cell fusion according to the general procedure of Kohler and Milstein (1975). MAb 7F6 was previously produced by immunizing mice with soluble ovine skeletal muscle troponin I (sTnI), which showed specificity to all ruminant sTnI including beef, sheep, and deer sTnI. The ascites fluid containing MAb 7F6 was generated from Auburn University Hybridoma Facility. The protocols for the extraction and purification of TnI from animal skeletal muscles, as well as the production of mouse MAb from ascites fluids, were as described by Chen and Hsieh (2002b). In contrast, a series of MAbs including 6F11 was elicited by mice immunized with crude soluble proteins extracted from cooked (in water bath at 100°C for 30min) ovine muscle with PBS-NaCl. These MAbs were propagated by in vitro bioreactor (Model CL350, Integra Bioscience, Inc. Ijamsville, MD) at the Hybridoma Laboratory, Florida
State University. The isotype of each MAb was determined by Mouse Monoclonal Antibody Isotyping Kit (ISO-2, Sigma) according to the manufacturer’s protocol (Appendix B.1).

The purification of MAbs 6F11 and 7F6 was performed using the Econo low pressure chromatography system and Affi-Gel protein A Monoclonal Antibody Purification System (MAPS II) kit (Catalog No. 153-6159, Bio-Rad) according to the procedures recommended by the manufacturer (Appendix B.2). Labeling of detection antibody 6F11 with NHS-CA-biotin was prepared according to the standard protocol (Harlow and Lane 1988) (Appendix B.3).

### 3.4 Indirect ELISA

The species specificity of each MAb was determined by indirect noncompetitive ELISA. Meat protein extracts were appropriately diluted in 0.06 M carbonate buffer (pH 9.6), and then 100 μL (containing 2 μg protein) of diluted extract was coated on each well of the microtiter plate (polyvinylchloride plate, Costar, Cambridge, MA). After incubation for 1 hour at 37°C or overnight at 4°C, the plate was washed three times with 250 μL/well of Washing Buffer (PBS containing 0.05% surfactant Tween 20, PBST) using a microplate washer (Model 1575, Bio-Rad) and then filled with 1% BSA-PBS (PBS buffer containing 1% BSA) to block nonspecific binding sites. Following 1-hour incubation and washing steps, 100 μL of diluted (1:3000 in 1% BSA-PBST) MAb 7F6 (1.541 mg/ml) or MAb 6F11 (1.452 mg/ml) immunoglobulin G (IgG) – alternatively, undiluted supernatant could be used – was added to the wells and incubated for another hour at 37°C. After the plate was washed three times with the Washing Buffer, 100 μL of diluted goat anti-mouse IgG peroxidase conjugate (Bio-Rad, 1:3000 in 1% BSA-PBST) was added to the wells followed by 1-hour incubation. The plate was washed again for 5 times, and finally, the bound enzyme activity was revealed by the addition of 100 μL of enzyme substrate (22 mg of ABTS and 15 μL of 30% hydrogen peroxide in 100 mL of 0.1 M phosphate-citrate buffer; pH 4.0) for color development. The enzyme reaction was stopped after 15 min by the addition of 100 μL of 0.2 M citric acid and the absorbance was measured at 415 nm using a microplate reader (Model 450, Bio-Rad).
3.5 SDS-PAGE and Western blot

The protein extracts of cooked samples were concentrated for electrophoresis using Microcon Centrifugal Filter Devices with cut-off nominal molecular weight of 10,000 Daltons (Model YM-10 membrane, Amicon, Beverly, MA). Sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Mini-Protean II electrophoresis unit (Bio-Rad) according to the method of Laemmli (1970) with some modifications. Briefly, the proteins were separated on a 4% stacking gel and a 12% separating gel, after running for about 40 min at a constant voltage of 200v, the gel was removed for electrophoretic transfer (Appendix B.4).

Following SDS-PAGE, Western blot was performed to determine the molecular weights of the antigenic proteins in meat extracts using MAb 7F6 and 6F11. The electrophoretic transfer (1 h at 100 V) of separated protein bands from SDS-PAGE gel to nitrocellulose membrane (Bio-Rad) was conducted using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the method of Towbin et al. (1979). After the transfer, the membrane was washed with 10x Tris Buffered Saline (TBS, Bio-Rad), and then blocked with the Blocking buffer (1% BSA in TBS) for 1 hour at room temperature. Next, the membrane was incubated with MAb 7F6 or MAb 6F11 IgGs diluted to 2.5-3 µg/ml in Antibody Buffer (TBS containing 1% BSA and 0.05% Tween 20) at 4°C overnight. The binding of the antibody with antigen was revealed by the addition of the second antibody – goat anti-mouse IgG - alkaline phosphatase (AP) conjugate (Bio-Rad, 1:3000 diluted in antibody buffer) followed by the addition of AP chromogen (BCIP/NBT, Bio-Rad). The color development was stopped by washing the membrane with DI water (Appendix B.5).

3.6 Sandwich ELISA

The optimal dilution of the capture MAb 7F6 and the detection MAb 6F11-biotin was determined by two-dimensional titration method. The highest sensitivity could be achieved by using 1:10,000 dilution of capture antibody 7F6 (1.541mg/ml) and 1:1000 dilution of detection antibody 6F11-biotin (1.36 mg/ml). 100 µL of properly diluted MAb 7F6 IgG, specific to ruminant (including beef, sheep, and deer) sTnI, was coated on each well of the microtiter plate. The plate was then incubated for 1 hour at 37°C or overnight at 4°C to
allow complete binding. After blocking with 1% BSA-PBS to saturate the remaining sites for protein binding on the microtiter plate, raw, cooked and autoclaved meat protein extracts were added to the wells (raw samples were diluted in the equal volume of 1% BSA-PBST containing 10mM EDTA; heat-processed samples were used without further dilution). After another 1-hour incubation, each well of the microtiter plate was added with 100 μL of properly diluted biotin-conjugated MAb 6F11. The addition of 100 μL per well of streptavidin-peroxidase conjugate (Sigma, 1:3000 diluted in 1% BSA-PBST) was followed, and the color development procedure was the same as described in indirect ELISA. Figure 1 showed the schematic of sandwich ELISA.

3.7 Statistical analysis

Each absorbance reading values in all assays were performed in triplicate, and each experiment was repeated three times. Means, standard deviations, coefficient of variations, and Student t-test were performed using Excel 2003 software (Microsoft Co., Seattle, Wash., U.S.A.). Significant difference was defined at \( p < 0.05 \).
4. RESULTS AND DISCUSSION

4.1 Selection of a pair of matched MAbs for sandwich ELISA

In the screening of hybridoma supernatants (containing MAbs) produced against cooked lamb extract, eight supernatants showing positive to cooked lamb antigen were tested for species specificity. As shown in Figure 2, supernatants 6F11, 1H4, 1H3, 1C6, 1E7, 5C3, 4G8, and 4B11 had strong positive reactions to cooked lamb with some cross-reactivity to beef or both beef and pork, but negative reactions to deer, horse and poultry (including chicken, turkey and duck). Because MAb 6F11 had the strongest reaction signal to cooked lamb sample compared to other MAbs, it was selected to be purified and labeled with biotin for the subsequent usage as the detection antibody in sandwich ELISA.

The detection antibody can be directly labeled with enzyme such as horseradish peroxidase (HRP) or can be conjugated to NHS-CA-biotin, which has an affinity of $10^{15} \text{M}^{-1}$ for the egg white protein avidin or its bacterial equivalent, streptavidin (Savage and others 1994). In this sandwich assay, biotin was chemically linked to the detection antibody 6F11, and commercial streptavidin-HRP (Sigma Chemical Co.) was used to reveal the color reaction.

The prerequisite for constructing a sandwich ELISA is that the capture Ab and the detection Ab bind to the same antigen simultaneously without interfering with each other (Figure 1). In these conditions, two such Abs can be considered a matched pair (Levieux and others 1995). Using sandwich assay format, a matching test was conducted in order to select a pair of matched MAbs. Seven unlabeled MAbs (1H4, 1H3, 1C6, 1E7, 5C3, 4G8, and 4B11) together with a previously developed MAb 7F6 (ruminant specific) were coated on the microtiter plate as the capture antibody. MAb 6F11 conjugated with biotin was used as the detection antibody. Sandwich ELISA based on each pair of MAbs strongly reacted with cooked lamb antigen, but slightly reacted with cooked beef (Figure 3). The successfulness of the sandwich assay verified that MAb 6F11 matched with each of other tested capture antibodies, and thus gave positive reaction signals. Since the highest signal was produced from the combination of 7F6 and 6F11-biotin, these two MAbs were selected
as the best matching pair to develop a sandwich ELISA that could specifically identify cooked ovine from other meat species.

MAbs 7F6 and 6F11 were further characterized by the indirect ELISA and Western blot, and the experimental conditions of the sandwich assay were optimized to increase the positive signal, and yet, keep the background as low as possible to achieve the highest sensitivity and specificity.

4.2 Characterization of MAbs by indirect ELISA

Isotypes of MAbs 7F6 and 6F11 were determined as IgG1 and IgG2a, respectively. MAbs 7F6 and 6F11 were characterized for species specificity using raw, cooked and autoclaved meat samples by indirect ELISA. Figure 4 indicates that MAb 7F6 was highly specific to ruminant meat extracts (including beef, lamb, and deer extracts) without cross-reactivity to other non-ruminant (pork, horse, chicken and turkey) meats tested. The immunoreactivity of MAb 7F6 towards ruminant proteins was not affected by heat-treatment, because cooked (100°C for 30min) and autoclaved (121°C for 30min) meat extracts exhibited identical ruminant specificity with raw meat samples to MAb 7F6.

Figure 5 shows that MAb 6F11 had different reactive patterns towards raw, cooked or autoclaved meat protein extracts. Among seven tested species (pork, beef, lamb, deer, horse, chicken, and turkey) with three treatment conditions (raw, cooked, and autoclaved), raw beef and lamb, cooked pork, beef and lamb, as well as autoclaved lamb exhibited positive reactivity to MAb 6F11. Autoclaved beef showed slight cross-reactivity. Comparing all the positive signals, lamb extracts exhibited much stronger reactivity than pork or beef extracts, indicating that MAb 6F11 had the higher affinity to lamb muscle proteins than to other meat proteins. The absorbance reading for cooked lamb extract was much higher than those of the raw and autoclaved lamb extracts, suggesting that appropriate heating temperature (cooking) might expose some inner epitopes which were concealed in the native protein molecules; severe heat-treatment (such as autoclaving) could cause further degradation of proteins or peptides, which might damage partial epitopes. Therefore, cooked lamb proteins contained much more available antigenic determinants (epitopes) which could interact with MAb 6F11, and consequently, a higher
reaction signal was obtained from cooked lamb extract than from raw or autoclaved lamb protein extracts.

4.3 Characterization of MAbs by Western Blot

MAbs 7F6 and 6F11 were further characterized by Western Blot. This technique tells the specific species reacted with the antibody, and also gives the information about the size of the proteins detected by the antibody.

4.3.1 Characterization of MAb 7F6 by Western Blot

Figure 6a, 6b and 6c shows the protein bands detected by MAb 7F6 in raw, cooked and autoclaved meat samples, respectively. As observed, only ruminant (including beef, lamb and deer) meat extracts yielded protein bands, indicating that MAb 7F6 was ruminant specific, which was in agreement with that verified by indirect ELISA. Furthermore, it is showed that the major protein bands detected by MAb 7F6 located around 24 kDa position, 1 – 3 weak protein bands (between 15 – 20 kDa) could also been observed in lamb and deer extracts. Raw, cooked and autoclaved meat samples exhibited exactly the same electrophoretic patterns, suggesting that the antigenic proteins detected by MAb 7F6 were not affected by the heat-treatment.

MAb 7F6 was produced by immunizing mice with ovine skeletal troponin I (sTnI), which is a soluble thermal-stable myofibrillar protein and can be extracted from skeletal muscles from different species by simple salt solution (Chen and Hsieh 2001; Chen and Hsieh 2002a, 2002b). Results from the SDS-PAGE showed that sTnI was corresponding to the 24 kDa band position (Chen and Hsieh 2002a, 2002b). Therefore, it could be concluded that MAb 7F6, produced against ovine sTnI, could react with all the ruminant sTnIIs, which existed in both raw and heat-treated ruminant protein extracts, and were located at the approximately 24 kDa band position in the gels or blots.

Several light protein bands ranging from 15 –20 kDa in the lamb and deer samples could be degraded peptides which carried the same sequential determinants as sTnI, and thus were detected by MAb 7F6 at the same time. These small sized protein bands (possibly peptides or protein subunits) appeared in both raw and heat-processed lamb and deer meat protein extracts, so it was likely that degradation of myofibril protein was due to the
postmortem enzymatic proteolysis during storage (Koohmaraie and others 1991; Redmond and others 2001; Volpelli and others 2005), and not from the cooking or autoclaving.

This study confirmed the heat-resistant nature of sTnI, which was consistent with a previous report that the heat-treatment (from 80°C for 30 min to 126°C for 120 min) did not affect the solubility and antigenicity of sTnI extracted from pork (Chen and Hsieh 2002a). The heat-stability of sTnI could be attributed to the durable structure of its sequential epitope (Mole 1994) and the plastic form of polypeptide chain (Pearlstone and others 1997).

4.3.2 Characterization of MAb 6F11 by Western Blot

The antigenic protein bands detected by MAb 6F11 had different patterns in raw (Figure 7a), cooked (Figure 7b), and autoclaved (Figure 7c) meat extracts. The pronounced visible bands only appeared in raw beef and lamb, cooked pork, beef and lamb, as well as autoclaved beef and lamb samples (Figure 7). Cooked horse and deer had several very weak bands around 10 and 15 kDa. These reactivity patterns corresponding to the species specificity of MAb 6F11 was consistent with that determined by indirect ELISA (Figure 5).

Considering the size of the antigenic proteins, raw beef and lamb had respective protein bands at about 30 kDa position (Figure 7a); cooked pork and lamb had a group of protein bands ranging from 10 – 30 kDa, and cooked beef had several bands between 10 – 15 kDa (Figure 7b). Autoclaved beef showed two 14 and 16 kDa bands, more intensive bands at 14, 16, and 19 kDa position appeared in autoclaved lamb extract (Figure 7c). A 24 kDa protein band (corresponding to the sTnI) could be identified in cooked lamb extract, but was very weak in cooked beef extracts.

It was more likely that the low molecular weight components detected by MAb 6F11 in cooked and autoclaved samples were heat-degraded protein subunits or peptides, which still carried the sequential epitope after degradation and thus maintained the reactivity with MAb 6F11 (Hsieh and others 1998). Because of the inherent mono-specificity of MAbs toward a single epitope on the antigen, the appearance of the multiple protein bands on the immunoblots implied that different antigens shared identical sequential epitope.

Unlike sTnI-specific MAb 7F6, MAb 6F11 was produced by immunizing mice with crude protein extract from cooked ovine skeletal muscles. The impure immunogen
contained various proteins, and some of them might carry the same antigenic determinant, which could explain the diversity of the protein bands appearing in the Western blot of MAb 6F11. In addition, cooked lamb extract was used as immunogen, and had the most immunogenic components, thus the most and strongest protein bands were generated in cooked lamb extract (Hsieh and others 1998).

4.3.3 Comparison of Western Blot with indirect ELISA

The species specificity and reactivity of MAbs 7F6 and 6F11 determined by indirect ELISA against raw, cooked and autoclaved meat samples were summarized in Table 1. The major antigenic proteins detected by MAb 7F6 or 6F11 in Western blots of raw, cooked and autoclaved meat samples were summarized in Table 2. Comparing Table 1 with Table 2, different immunoassay formats, indirect ELISA and Western blot, exhibited identical species specificity for each MAb. The species with high reactivity in indirect ELISA had correspondingly intensive protein band(s) in Western blot.

It is well known that the epitope may be dependent upon the presence of a specific 3-dimensional structure (conformational epitope), or may correspond to a simple primary sequence region (linear epitope) (Wessels and others 1990; Huang and Honda 2006). In Western blot, antigenic proteins are denatured by SDS. In indirect ELISA, antigens are coated on the solid phase (surface of the microtiter plate), the fixing or embedding process may change the conformation of the antigen molecules. That means that the antigenic proteins used in both Western blot and indirect ELISA have been denatured. Therefore, the same results, i.e. the same reactive patterns, might be obtained from these two different immunoassay formats.

4.4 Species specificity by sandwich ELISA

Results from the matching test demonstrated that MAbs 7F6 and 6F11 based sandwich ELISA could successfully identify cooked ovine from other species (Figure 3). However, the absorbance reading of cooked lamb was less than 0.7. The sensitivity of the assay needed to be increased by optimizing the experimental conditions, such as dilution of
the antigen and two antibodies, selection of blocking buffer, antigen and antibody buffer, as well as incubation time and color development time.

As shown in Figure 8, after optimization, the absorbance reading of cooked lamb extract was increased from 0.632 obtained in the matching test (Figure 3) to 2.254; in the meanwhile, the background absorbance readings from other cooked non-ovine species (including beef, pork, deer, horse, chicken and turkey) were controlled under 0.2. The optimal dilutions for the capture antibody 7F6 (1.541 mg/ml) and the detection antibody 6F11-biotin (1.360 mg/ml) were 1:10,000 and 1:1000, respectively. In the matching test without optimization, the dilutions for MAbs 7F6 and 6F11-biotin were 1:6000 and 1:5000, respectively. This demonstrated that optimization of experimental conditions was critically important in developing a successful ELISA system. Table 3 summarizes the optimal experimental conditions.

Theoretically, based on the species specificity of both MAbs (Table 1), ruminant specific MAb 7F6 could capture raw, cooked and autoclaved bovine, ovine and deer proteins, while non-specific porcine, equine, chicken, and turkey proteins would be removed from the microtiter plate after the washing step. Biotin conjugate MAb 6F11 would then detect bovine and ovine, because 6F11 could not react with deer proteins. In addition, according to the immunoreactivity of MAb 6F11 obtained from indirect ELISA, lamb had much stronger affinity with 6F11 than beef (the absorbance readings of lamb were 3–4 times higher than beef for heat-treated samples), and cooked lamb had higher reactivity than autoclaved lamb (Figure 5); likewise, in Western blot analysis, lamb extract yielded stronger protein bands than beef extract, and cooked lamb had more bands than autoclaved lamb (Figure 7b). Therefore, it was expected that this sandwich assay should display: (1) bovine and ovine specificity for raw meat extracts; and (2) ovine specificity with cross-reaction to bovine for heat-processed meat samples, and higher reaction signals from cooked samples.

Indeed, the optimized sandwich ELISA showed expected bovine and ovine specificity for raw meat samples, but cross-reaction with raw deer and equine was unexpected. For heat-treated meat samples, ideal mono specificity to ovine meat was achieved without cross-reaction to other species, and cooked ovine had much higher absorbance reading than autoclaved ovine (2.254 versus 0.887).
For heat-treated meat samples, ovine extracts displayed expected reactivity patterns, but beef extracts lost antigenicity. One reason was optimization, because cooked beef still showed some cross-reaction (data not shown) without optimal dilutions for both Abs and optimal color development time. Another reason was that the major antigenic proteins recognized by both MAb 7F6 and 6F11 were identical for lamb samples but different for beef samples. As mentioned previously, a successful sandwich ELISA requires double Abs recognizing distinct epitopes on the same antigen. MAb 7F6 reacted with 24-kDa protein in beef extracts (Figure 6b and 6c); however, this 24-kDa band was very weak in cooked beef and lacking in autoclaved beef in the immunoblot of 6F11 (Figure 7b and 7C). That means that only small amounts of common antigens (24 kDa protein) could be detected by both Abs in cooked beef, and no common antigenic protein reacted with both Abs in autoclaved beef. That could explain the low absorbance reading for cooked beef sample, and the loss of reactivity for autoclaved beef extract. In contrast, cooked lamb had one 24-kDa band and two 15 – 20 kDa bands which appeared on both blots of 7F6 and 6F11, and autoclaved lamb showed the same 19 kDa band in both blots, suggesting that cooked lamb had more common antigens recognized by both MAbs, thus produced higher absorbance reading than autoclaved lamb.

For raw meat samples, although bovine and ovine had the expected positive reactivity, the protein bands which appeared on the blots of MAbs 7F6 and 6F11 in beef and lamb samples were different, i.e. a 24 kDa protein in the blot of 7F6 (Figure 6a) and an approximately 30 kDa protein in the blot of 6F11 (Figure 7a). In addition, 7F6 had no reaction with horse; 6F11 could not react with either deer or horse. It was difficult to explain the cross-reactivity with deer and horse using the results of Western blot.

Inconsistency of the results for raw meat samples between sandwich ELISA and indirect ELISA or immunoblots could be attributed to the difference in these immunoassay formats. As mentioned before, in the Western blot, antigens are denatured by detergent – SDS; in indirect ELISA, antigens may be altered by immobilization. In sandwich ELISA, antigenic molecules are neither affected by SDS nor changed by immobilizing procedures. Therefore, antibodies that may fail to detect antigens in Western blot or indirect ELISA due to the conformational alteration of the target molecule, but retain effectiveness for antigen binding in sandwich ELISA since antigenic proteins maintain complete 3-D structure.
On the other hand, cooked and autoclaved meat proteins were already denatured through heat-treatment. Denatured antigens were used in all three types of immunoassay formats – Western blot, indirect ELISA, and sandwich ELISA – so consistent results could be obtained among them.

This study demonstrated that the mono-specific sandwich ELISA can be successfully developed by using two MAbs that have broad specificity, because undesired cross-reactivity can be eliminated owing to lack of identical antigenic molecules being bound by both MAbs simultaneously, and thus specific reactivity is achieved on account of specific antigens being recognized by double antibodies. In addition, some high background signals can be well controlled by optimization of the experimental conditions to obtain the best sensitivity and specificity.

4.5 Cross-reactivity with non-meat proteins by sandwich ELISA

To examine the cross-reactivity with non-meat proteins, cooked salmon, ovine blood, bovine serum albumin (BSA), soy proteins, egg albumin, milk proteins and gelatin, were tested by this sandwich assay, since some of these proteins might contaminate food products and some might be used as common food additives. As shown in Figure 9, only soy proteins displayed slight cross-reaction in this assay (absorbance reading: 0.246), other proteins showed no cross-reactivity. As described in “sample preparation” section, the final content for each of the non-meat proteins was 10g/120 ml, whereas cooked meats were extracted in 2-fold (wt/vol) PBS-NaCl solution, (i.e. 10g/20 ml). That means, the background reading of 0.246 from soy proteins was yielded when 6 parts of non-ovine meat adulterated with 1 part of soy protein concentrate. If the content of soy proteins was reduced (less than 1: 6), the background reading was less than 0.2 (data not shown). In another words, the slight cross-reactivity with soy proteins only occurred when food products contained a high content (more than 10%) of the soy ingredient.

In conclusion, MAbs 7F6 and 6F11-based sandwich ELISA could effectively identify cooked ovine muscle proteins from non-ovine meat (pork, beef, deer, horse, chicken and turkey) without cross-reactivity to most common food proteins. Nevertheless, this assay would be less sensitive when being used for the detection of lamb meat in autoclaved muscle food products.
4.6 Detection limits of laboratory-adulterated meat samples

The sensitivity of this sandwich ELISA was assessed using laboratory-adulterated cooked meat mixtures including lamb-in-pork, lamb-in-beef, and lamb-in-chicken at 0 – 10% levels. As shown in Figure 10a-c, the assay successfully detected low levels of cooked lamb in pork, beef, or chicken mixtures. The baseline (0% adulteration level) reading for lamb-in-beef was higher (0.185) than lamb-in-pork (0.121) and lamb-in-chicken (0.129), suggesting that the beef gave a higher background reading than pork and chicken. The same observations were shown in the sandwich ELISA for determining the species specificity (Figure 8). The absorbance readings for lamb-in-chicken samples increased more rapidly with the increase of the lamb meat adulteration levels than did the absorbance readings for lamb-in-pork and lamb-in-beef samples. For example, the absorbance readings of 1% adulteration level were 0.547, 0.208, and 0.248 for lamb in chicken, pork and beef, respectively. Correspondingly, the slope of the detection curve for lamb-in-chicken was greater than those for lamb-in-pork and lamb-in-beef. Therefore, to detect lamb meat in cooked meat mixtures by this sandwich ELISA, using chicken meat as the matrix could give the highest sensitivity compared to using pork or beef meat as the matrix.

The sensitivity of this assay was also reflected by the detection limit, which is defined as the lowest adulteration level at which the absorbance reading is significantly (p<0.05) higher than the baseline. Determined by Student t-test, the detection limits for lamb-in-pork, lamb-in-beef, and lamb-in-chicken were 0.5%, 0.5%, and 0.25%, respectively (Figure 10). This further demonstrates the high sensitivity of the assay for the detection of lamb in cooked chicken mixture.

As shown in Figure 10a and 10b, the baseline reading of beef was higher than pork, but the increase of the readings for lamb-in-pork was greater than lamb-in-beef. At the 10% adulteration level, the reading of the lamb-in-pork (0.721) was higher than the reading of the lamb-in-beef (0.660) (p<0.05). These results suggest that the sensitivity of the assay was better for detection of lamb meat in pork than in beef.

Other commonly used methods for the identification of cooked lamb were DNA-based polymerase chain reaction (PCR) techniques and the PAb-based ELISA approach. PCR techniques have been widely studied for meat speciation, and could provide adequate
sensitivity in analysis of heat-processed meat samples. By amplification of sheep satellite I DNA sequence and analysis the PCR products on 4% agarose gel electrophoresis, Chikuni and others (1994) could detect 10 pg of sheep DNA. Although cooking had the negative impact on the PCR products, sheep DNA could still be differentiated after being heated to 120°C using this method. Matsunaga and others (1999), utilizing different primers in the mitochondrial cytochrome b genes, developed a multiplex PCR, which could detect 250 pg of sheep DNA after being heated to 121°C. Another multiplex PCR method, also based on primers in the mitochondrial gene, was developed for the qualitative detection of pork, beef, sheep and goat meat autoclaved at 121°C and 15 lb/cm² for 20 min. The detection limit was determined using binary muscle-muscle mixtures (wt/wt), showing that 1% of sheep-in-pork or goat-in-pork could be identified in this assay (Rodriguez and others 2004).

A method of fluorescence PCR – restriction fragment length polymorphism (RFLP) was designed for identifying cooked (100°C for 30 min) and autoclaved (121°C for 30 min) goat, pork and beef, and each species in binary mixtures could be semi-quantified at 1% (wt/wt) level (Sun and Lin 2003). Lopez-Andreo and others (2005) designed a real-time PCR system for identification and quantitation of bovine, porcine, ovine, chicken, turkey, and ostrich DNA in complex sample mixtures (two to four species DNA extracts, vol/vol). This assay could quantitatively detect more than 5% of cattle or lamb, but the accuracy of the assay decreased as the samples contained less than 10% beef or pork.

The previously mentioned PCR methods could discriminate ovine meat in heat-processed meat mixtures with the lowest detection limit of 1% (wt/wt), and some of these methods could give a semi-quantitative measurement despite the low accuracy for analysis of low level adulterant. However, MAbs 7F6 and 6F11-based sandwich ELISA could achieve lower detection limits, 0.5% for lamb-in-pork or lamb-in-beef, and 0.25% for lamb-in-chicken. Moreover, MAb-based sandwich ELISA can be developed into a highly accurate, effective, and quantitative method for meat speciation.

A well-recognized drawback for PCR methods is that they are susceptible to contamination, and thus delicate facilities and extreme caution is needed. Whereas, ELISA techniques are rugged and suitable for field test.

Some commercial ELISA kits such as ELISA-TEK™ Cooked Meat Species Kits (ELISA Technologies, Gainesville, FL) and BioKits (Cooked) Species Identification Kits.
(Tepnel BioSystems Limited, U.K.) can qualitatively detect sheep in cooked or even autoclaved foods with detection limits of 1% and < 2%, respectively. One reported sandwich ELISA could detect 0.13% sheep in cooked meat extract mixture (Andrews and others 1992). However, all of these methods utilize PAbs or antiserum.

By far, MAb-based ELISA methods for identifying heat-processed ruminant and mammalian meats have been reported (Hsieh and others 1998; Chen and others 2004). These ELISA systems could detect cooked ovine meat, but they are not mono-species specific. There is no reported MAb-based ELISA that can identify single ovine species from cooked meat mixtures. Therefore, this study is the first MAb-based sandwich ELISA that enables the simple and reliable detection of ovine muscle tissue in cooked food products.

4.7 Effect of heat treatment and freezing storage

Figure 8 showed that cooked lamb had a higher reaction signal than autoclaved lamb in the optimized sandwich ELISA. To further assess the effectiveness of the assay in analyzing meat products treated with different heating times and temperatures, the second batch of samples using ground lamb which had been stored at -80ºC for six months was prepared with six kinds of heat treatment conditions: cooking at 100ºC for 15, 30, and 60 min, and autoclaving at 121ºC/1.2 bar for 15, 30 and 60 min.

As shown in Figure 11, the absorbance readings for heat-treated lamb meat extracts decreased rapidly with an increase in heating time and temperature/pressure. Lamb autoclaved for only 15 min had a much lower absorbance reading (0.270) than lamb cooked for 60 min (0.735), suggesting that temperature and pressure had a more negative influence on the antigenicity of ovine proteins than the duration of heating.

In this sandwich ELISA, the absorbance readings for lamb meat cooked and autoclaved for 30 min were 1.851 and 0.190, respectively (Figure 11). However, in initial sandwich assay using the lamb samples treated with the same conditions, the readings for cooked and autoclaved lamb were 2.254 and 0.887 (Figure 8). The only difference between the two batches of samples was that the batch #2 samples were prepared using ground lamb meat which had been frozen for 6 months at – 80ºC, whereas the batch #1 samples were
extracted immediately after purchasing and grinding without frozen storage. Therefore, a total of four lamb samples (cooked and autoclaved for 30min from two batches) were chosen for further testing in the same microtiter plate. As shown in Figure 12, after 6 months of frozen storage, both cooked and autoclaved lamb meat extracts exhibited reduced reactivity, and the reduced reactivity was more pronounced for autoclaved lamb than for cooked lamb extract.

It has been demonstrated that the structural changes of myofibrillar proteins during the postmortem storage of meat are caused by muscle proteinases, especially the calpains (calcium-activated neutral proteases) and cathepsins (lysosomal proteases) (Etherington and others 1990; Ladrat and others 2004). It has also been reported that the calpain/calpastatin system is responsible for the proteolytic degradation of lamb myofibrillar proteins during postmortem (Redmond and others 2001). Other scientists observed that calpains play a significant role in proteolysis of myofibrillar proteins when goat muscles were stored at 5°C (Nagaraj and others 2002, 2005). Furthermore, the effects of frozen storage (-15°C) up to 120 days on the properties of myofibrils, muscle proteinases (cathepsins and calpains) and their endogenous inhibitors in goat muscles were also investigated by Nagaraj and Santhanam (2006). The authors found that calpains, not cathepsins, might cause the structural changes of myofibrillar proteins during frozen storage of the goat muscle, although, these changes were quite small in the electrophoresis pattern of myofibrils (Ikeuchi and others 1980).

The ground lamb meat used for the preparation of the second batch of samples was stored at an ultra low temperature (-80°C) for a long duration (about 180 days). The degradation activities of the lamb muscle proteins might be caused by the proteolytic system as observed by Nagaraj and Santhanam (2006). The degradation of lamb myofibrillar proteins was verified through the loss of their antigenicity, which subsequently reduced the reactivity of lamb samples in the sandwich assay.

In conclusion, either severe heat treatment such as high temperature and pressure, or long storage time even at an extra low freezing temperature could cause the degradation of myofibrillar proteins, which might destroy the epitope and affect its ability to interact with an antibody. Thus, the immunoreactivity of the target antigen would be reduced or even disappeared.
4.8 Validation of the sandwich assay

The effectiveness of this MAb-based sandwich ELISA had been evaluated by determining the detection limit and cross-reactivity. To further validate and describe this sandwich assay, intra- and inter-assay variability (also known as coefficient of variation, CV) was calculated. The less the variability, the better the reproducibility, which means the ability of the assay to duplicate results in repeat determinations.

Percent coefficient variation (%CV) is often reported on a scale of 0 to 100%, and is computed by dividing the standard deviation by the mean of the replicate determinations and multiplying by 100. The higher the CV, the higher the variability. Usually the reproducibility of an assay is considered to be good if the CV is less than 10%.

In this study, intra-assay CV between replicate determinations in the same assay and inter-assay CV between replicate determinations from different groups were calculated. As shown in Table 4, for lamb-in-pork, lamb-in-beef, and lamb-in-chicken samples with adulteration levels ranging from 0% to 10%, the average intra-assay CVs were 3.4%, 3.5%, and 3.2%; and the average inter-assay CVs were 6.0%, 4.7%, and 5.9%, respectively.

The increased regulation of food products in modern society requires analytical methods which are easy to perform, sensitive, specific and relatively inexpensive. This MAb-based sandwich ELISA that has been newly developed fulfills all these requirements, and its effectiveness in detecting ovine muscle proteins in cooked meat mixture was validated. It thus has potential to be developed into a routine screening method for the identification and authentication of ovine meat origin in food products.
5. CONCLUSION

Based on the matching pair of MAbs 7F6 and 6F11-biotin, a sandwich ELISA was developed to detect ovine meat in cooked meat mixtures. This assay is highly sensitive and specific without cross-reactivity with other meat species such as pork, beef, deer, horse, chicken and turkey. In addition, there was no apparent cross-reaction with other non-meat proteins such as egg albumin, gelatin, milk protein, fish protein, sheep blood, and bovine serum albumin, which might be either commonly used as food additives or present in contaminated food products. Only soy proteins exhibited slight cross-reactivity.

The detection limits for cooked lamb-in-pork, lamb-in-beef and lamb-in-chicken were 0.5%, 0.5% and 0.25%, respectively. Autoclaved meat had reduced reaction signals. Therefore, this assay was less sensitive in analyzing autoclaved meat samples. Storage of lamb meat for a long time (6 months) even at ultra low temperature (-80°C) also caused reduced reactivity. For lamb-in-pork, lamb-in-beef, and lamb-in-chicken samples at adulteration levels ranging from 0% to 10%, the average intra-assay CVs were 3.4%, 3.5%, and 3.2%; average inter-assay CVs were 6.0%, 4.7%, and 5.9%, respectively.

This is the first report of a MAb-based sandwich ELISA that could effectively detect trace amounts of ovine muscle tissue in cooked meat mixtures. This assay is simple, rapid, and highly sensitive. It has the potential to be developed into a quantitative analytical method and commercial field-test ELISA kits for large-scale screening of meat products to detect and prevent adulteration.
APPENDIX A. ABBREVIATIONS

ABTS: 2,2'-azino-di-3-ethyl-benothiazoline-6-sulfonic acid
AP: Alkaline Phosphatase
BCIP/NBT: 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium
BSA: bovine serum albumin (BSA)
1%BSA-PBS (Blocking buffer for ELISA): PBS buffer containing 1% BSA
1%BSA-PBST (Antibody buffer for ELISA): PBS containing 0.05% Tween 20 and 1% BSA
1%BSA-TBS (Blocking Buffer for Western blot): 1%BSA in TBS
1%BSA-TBST (Antibody Buffer for Western blot): 1%BSA in TBST
CB: 0.06 M carbonate buffer (pH 9.6)
CB-EDTA: 0.06 M carbonate buffer containing 10 mM EDTA (pH 9.6)
DI water: deionized water
DMF: N, N-Dimethyl Formamide
EDTA: ethylenediaminetetraacetic acid
HRP: horseradish peroxidase
IgG: immunoglobulin G
MAPS: Monoclonal Antibody Purification System
PBS: 0.01 M phosphate buffer saline (pH 7.2)
PBST (Washing Buffer for ELISA): PBS solution containing 0.05% Tween 20.
PBS-NaCl: 0.01 M phosphate buffer saline containing 0.5M sodium chloride (pH 7.2)
TBS: Tris Buffered Saline
TBST (Washing Buffer in Western blot): 0.05% (v/v) Tween-20 in TBS
APPENDIX B. PROTOCOLS AND PROCEDURES

Protocol 1. Determining the isotypes of MAbs by mouse MAb isotyping kit (Sigma)

For determination the isotypes of mouse MAbs 7F6 and 6F11 which were in the form of culture supernatants, the most suitable assay system is “Capture ELISA” according to the guide of the manufacture. The procedures for Capture ELISA were as described in the “ISO-2 Directions for Use” (Sigma 2002) with some modifications:

1. The isotype specific antibodies were 1:1,000 diluted in PBS.
2. 0.1 ml of each of the diluted antibodies was added into 2 wells of a microtiter plate.
3. The plate covered with Parafilm was incubated for 1 hour at 37 °C.
4. After removing the coating solution, the plate was washed 3 times with Washing Buffer (PBST).
5. 0.1 ml of the sample to be tested was added into each of the wells (culture supernatant undiluted was used).
6. The plate was incubated at room temperature for 1 hour.
7. The plate was washed 3 times with Washing Buffer.
8. Peroxidase labeled Goat Anti-Mouse IgG (Fab Specific) antibody was 1:600 dilute in the Washing Buffer.
9. 0.1 ml of the enzyme conjugated antibody was added into each well.
10. The plate was incubated at room temperature for 30 minutes.
11. The substrate was prepared as follows:
    a. ABTS was dissolved in 0.1M phosphate-citrate buffer at the ratio of 22 mg per 100ml.
    b. 30% hydrogen peroxide solution was added into the ABTS substrate buffer at the ration of 15 µl per 100 ml.
12. At the end of the 30 minute incubation, the plate was washed 5 times with Washing Buffer.
13. 0.1 ml of the freshly prepared substrate solution was added into each well.
14. The plate was incubated at room temperature for 20-30 minutes. Green color developed in a well indicates a positive result.

15. The reaction was stopped by addition of 100 µl of 0.2M citric acid to each well.

16. The plate could be read visually or by a microplate reader at 415 nm.

**Protocol 2. Purification of MAb**

1. The ascites fluid containing MAb 7F6 was centrifuged (3,220 x g) at 4°C for 30 min, the supernatant was diluted 1:3 (1 part ascites fluid plus 2 parts buffer) in Binding Buffer (Catalog No. 153-6161, Bio-Rad. pH 9.0 ± 0.2);

2. Large amount of tissue culture of 6F11 from bioreactor was prepared by adding the dry MAPS II Binding Buffer salts directly to the sample (31.4 g buffer salts/100 ml sample);

3. All the samples were filtered through a Sterile Syringe filter (Osmonics 0.45 μm) before loading to the Protein A cartridge.

4. The Protein A cartridge was connected to the Econo System (Bio-Rad) and the cartridge was equilibrated with 5 bed volumes of Binding Buffer;

5. The prepared sample was loaded on the column at a flow rate of 0.5 – 1 ml/min;

6. The column was washed with 15 bed volumes of Binding Buffer at a flow rate of 1 ml/min;

7. The antibody was eluted with 5-15 bed volume of Elution buffer (Catalog No. 153-6162, Bio-Rad. pH 3.0 ± 0.2), and then purified IgG was salted out by adding 1:1 (vl/vl) saturated ammonium sulfate solution;

8. Antibody solution was set at room temperature for 30 minutes, then centrifuged (3,220 x g) at 4°C for 20 minutes;

9. The supernatant was discarded, the pellet (IgG) was dissolved in 3 ml of PBS buffer;

10. The IgG solution was dialyzed against PBS buffer over 24 hours with changing the buffer 3 times;
11. The concentration of purified IgG solution was determined by UV spectrophotometer (SmartSpec 3000, Bio-Rad) at 280 nm.

**Protocol 3. Labeling of MAb with biotin**

1. The purified IgG 6F11 was dialyzed against 0.1 M NaHCO$_3$ at 4°C overnight and the dialysis buffer was changed at least 4 times;
2. The concentration of the dialyzed MAb was determined using UV assay;
3. 10 mg NHS-CA-biotin was dissolved in 1 ml DMF at 10 mg/ml;
4. 50 µg NHS-CA-biotin in DMF was added to each mg of dialyzed MAb, and then the solution was incubated at room temperature for 60 min;
5. The solution was dialyzed in 10 mM PBS at 4°C overnight and the dialysis buffer was changed at least 4 times;
6. The concentration of the dialyzed biotin conjugated 6F11 was determined using UV assay.

**Protocol 4. Procedures for SDS-PAGE**

1. The glass plate sandwiches was assembled according to the guide of the manufacture;
2. The glass sandwich was filled with 12% separating gel and 4% stacking gel;
3. Sample buffer was prepared by mixing 0.5M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 25% (vol/vol) glycerol, 0.01 % (wt/vol) bromophenol blue, and 5% (vol/vol) β-mercaptoethanol which was added prior to use;
4. The protein extracts were diluted at least 1:2 with sample buffer;
5. 3 – 4 µg of protein extract was loaded to each well of the gel;
6. The gel was run at a constant voltage setting of 200v for approximately 40 min at room temperature;
7. The gel was removed for electrophoretic transfer.

**Protocol 5. Procedures for Western blot**

1. The gel was rinsed with DI water;
2. The gel was equilibrated for 5-10 minutes in transfer buffer (10X Tris/Glycine Buffer, Bio-Rad) before blotting to remove electrophoresis salts and detergents;
3. The nitrocellulose membrane was cut to the dimensions of the gel and wetted by slowly sliding it at 45° into the transfer buffer for 15-30 min;
4. The pre-cut filter paper and Fiber Pads was soaked in the transfer buffer avoiding entrapment of air bubbles;
5. The gel holder cassette was assembled to complete the blotting sandwich;
6. The electrophoretic transfer was performed at 100V/350 mA for 1 hour;
7. After the transfer, the membrane was washed with TBS solution (Bio-Rad), and then the membrane was blocked with the blocking buffer (1% (w/v) BSA in TBS) for 1 hour at room temperature;
8. The membrane was washed twice with Washing Buffer (0.05% (v/v) Tween-20 in TBS);
9. The membrane was incubated with MAb 7F6 or 6F11 1:500 diluted in antibody buffer (1% (w/v) BSA in TBST) for 2h at room temperature or overnight at 4°C.
10. The membrane was washed 4 times with TBST;
11. The membrane was incubated with second antibody – Goat Anti-Mouse IgG (H+L)-AP Conjugate (Bio-Rad) (1:3000 diluted in antibody buffer) for 1h at room temperature;
12. The membrane was washed 4 times with TBST and 3 times with TBS prior to color development;
13. The membrane was stained by soaking it in 15 ml of color development buffer with addition of 150 µl of AP reagent A and 150 µl of reagent B (or similar ratio, Bio-Rad) at room temperature;
14. The staining was stopped by washing the membrane with DI water for about 10 min to remove residual color development solution;
15. The membrane was dried on filter paper.
APPENDIX C. TABLES AND FIGURES

Table 1. Species specificity and reactivity of MAbs 7F6 and 6F11. The species specificity and reactivity of MAbs 7F6 and 6F11 were determined by indirect ELISA against protein extracts from raw, cooked and autoclaved pork (P), beef (B), lamb (L), horse (H), deer (D), chicken (C), and turkey (T). The reactivity was defined as very strong (++++, when the absorbance reading was above 1.5), strong (+++, absorbance reading between 1.0 – 1.5), and moderate (++, absorbance reading between 0.5 – 1.0).

<table>
<thead>
<tr>
<th>MAb</th>
<th>Subclass</th>
<th>Samples</th>
<th>P</th>
<th>B</th>
<th>L</th>
<th>H</th>
<th>D</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>7F6</td>
<td>IgG1</td>
<td>Raw</td>
<td>–</td>
<td>+++</td>
<td>++++</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cooked</td>
<td>–</td>
<td>+++</td>
<td>++++</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoclaved</td>
<td>–</td>
<td>+++</td>
<td>++++</td>
<td>–</td>
<td>+++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6F11</td>
<td>IgG2a</td>
<td>Raw</td>
<td>–</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cooked</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoclaved</td>
<td>–</td>
<td>Trace</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2. Western blot analysis of meat protein extracts with MAbs 7F6 and 6F11. The molecular weights of the major antigenic proteins detected by MAbs 7F6 and 6F11 in Western blots were estimated according to the prestained protein standards. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey. MAb 7F6 (1.541mg/ml, 1:500) and MAb 6F11 (0.416mg/ml, 1:167) were diluted in 1%BSA-TBST.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Samples</th>
<th>P</th>
<th>B</th>
<th>L</th>
<th>H</th>
<th>D</th>
<th>C</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>7F6</td>
<td>Raw</td>
<td>–</td>
<td>~24 kDa</td>
<td>~24 kDa</td>
<td>~25 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>–</td>
<td>~24 kDa</td>
<td>~24 kDa</td>
<td>~25 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Autoclaved</td>
<td>–</td>
<td>~24 kDa</td>
<td>~24, 18,19 kDa</td>
<td>~25 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6F11</td>
<td>Raw</td>
<td>–</td>
<td>~30 kDa</td>
<td>~30 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>~30 kDa, 12-18 kDa</td>
<td>10-15 kDa</td>
<td>10-30 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Autoclaved</td>
<td>–</td>
<td>~14, 16 kDa (weak)</td>
<td>~14, 16, 19 kDa</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>
Table 3. Optimized experiment conditions for sandwich ELISA.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Sandwich ELISA</th>
<th>Optimized experiment conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture MAb 7F6 (1.541mg/ml)</td>
<td>1:10,000 diluted in PBS</td>
<td></td>
</tr>
<tr>
<td>Detection MAb 6F11-biotin (1.36 mg/ml)</td>
<td>1:1000 diluted in 1%BSA-PBST</td>
<td></td>
</tr>
<tr>
<td>Raw meat samples</td>
<td>1:1 diluted in 1% BSA-PBST containing 10mM EDTA</td>
<td></td>
</tr>
<tr>
<td>Cooked meat samples</td>
<td>Undiluted</td>
<td></td>
</tr>
<tr>
<td>Autoclaved meat samples</td>
<td>Undiluted</td>
<td></td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>1:3000 diluted in 1%BSA-PBST</td>
<td></td>
</tr>
<tr>
<td>Incubation between each step</td>
<td>37ºC for 1 hour</td>
<td></td>
</tr>
<tr>
<td>Color development time</td>
<td>Room temperature for 15 min</td>
<td></td>
</tr>
<tr>
<td>Enzyme substrate</td>
<td>22 mg of ABTS and 15 µL of 30% hydrogen peroxide in 100 mL of 0.1 M phosphate-citrate buffer; pH 4.0</td>
<td></td>
</tr>
<tr>
<td>Stop solution</td>
<td>0.2 M citric acid</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Intra- and inter-assay coefficient of variation (CV). Intra- and inter-assay CVs for lamb-in-pork, lamb-in-beef, and lamb-in-chicken were calculated according to the equation: CV = (standard deviation / mean) x 100%. Intra-assay CV was calculated using replicate determinations in the same plate, and inter-assay CV was calculated using replicate determinations from assays conducted in different date.

<table>
<thead>
<tr>
<th>Intra-assay CV</th>
<th>Lamb-in-pork</th>
<th>Lamb-in-beef</th>
<th>Lamb-in-chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>1.7 %</td>
<td>2.8 %</td>
<td>7.1 %</td>
</tr>
<tr>
<td>0.25 %</td>
<td>–</td>
<td>–</td>
<td>2.2 %</td>
</tr>
<tr>
<td>0.5 %</td>
<td>1.2 %</td>
<td>1.8 %</td>
<td>2.3 %</td>
</tr>
<tr>
<td>1 %</td>
<td>3.0 %</td>
<td>5.9 %</td>
<td>3.3 %</td>
</tr>
<tr>
<td>2 %</td>
<td>5.4 %</td>
<td>1.4 %</td>
<td>1.8 %</td>
</tr>
<tr>
<td>4 %</td>
<td>6.6 %</td>
<td>5.0 %</td>
<td>2.3 %</td>
</tr>
<tr>
<td>10 %</td>
<td>2.6 %</td>
<td>4.1 %</td>
<td>3.6 %</td>
</tr>
<tr>
<td>Average intra-assay CV</td>
<td>3.4 %</td>
<td>3.5 %</td>
<td>3.2 %</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Inter-assay CV</th>
<th>Lamb-in-pork</th>
<th>Lamb-in-beef</th>
<th>Lamb-in-chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>8.3 %</td>
<td>2.6 %</td>
<td>9.1 %</td>
</tr>
<tr>
<td>0.25 %</td>
<td>–</td>
<td>–</td>
<td>3.1 %</td>
</tr>
<tr>
<td>0.5 %</td>
<td>1.2 %</td>
<td>3.6 %</td>
<td>8.9 %</td>
</tr>
<tr>
<td>1 %</td>
<td>4.0 %</td>
<td>6.7 %</td>
<td>2.3 %</td>
</tr>
<tr>
<td>2 %</td>
<td>7.5 %</td>
<td>5.0 %</td>
<td>3.0 %</td>
</tr>
<tr>
<td>4 %</td>
<td>7.5 %</td>
<td>6.5 %</td>
<td>7.6 %</td>
</tr>
<tr>
<td>10 %</td>
<td>7.5 %</td>
<td>3.7 %</td>
<td>7.1 %</td>
</tr>
<tr>
<td>Average inter-assay CV</td>
<td>6.0 %</td>
<td>4.7 %</td>
<td>5.9 %</td>
</tr>
</tbody>
</table>
Figure 1. Schematic of the sandwich ELISA. MAb 7F6 was used as the capture antibody, MAb 6F11 conjugated with biotin was used as the detection antibody. The color was revealed by the addition of streptavidin-peroxidase conjugate and enzyme substrate.
**Figure 2.** Screening test of MAbs against cooked ovine proteins. Cooked meat samples were diluted in 0.06 M carbonate buffer (CB), and coated on the microtiter plate at 2µg/100µl. Undiluted cultural supernatants of MAbs were used. P: pork, B: beef, L: lamb, D: deer, H: horse, C: chicken, T: turkey. Blk: blank (background reading, no antigen and antibody added in the well). Each data was mean of triplicate (repeated in three wells in one plate) with SD bar shown.
Figure 3. Selection of capture antibody by sandwich ELISA. Biotin conjugated MAb 6F11 (1.36 mg/ml, 1:5000 diluted in 1%BSA-PBST) was used as the detection antibody. All the capture MAbs were undiluted supernatant, except that MAb 7F6 IgG (1.541mg/ml) was 1:6000 diluted in 1%BSA-PBST. P: pork, B: beef, L: lamb, C: chicken, Blk: blank. Each data was mean of triplicate with SD bar shown.
Figure 4. Species specificity of MAb 7F6 by indirect ELISA. Raw meat samples were diluted in CB-EDTA, cooked and autoclaved samples were diluted in CB, the amount of antigens coated on the plate was 2 µg/100µl. MAb 7F6 (1.541mg/ml) was 1:10,000 diluted in 1%BSA-PBST. P: pork, B: beef, L: lamb, D: deer, H: horse, C: chicken, T: turkey, Blk: blank. Each data was mean of triplicate with SD bar shown.
Figure 5. Species specificity of MAb 6F11 by indirect ELISA. Raw meat samples were diluted in CB-EDTA, cooked and autoclaved samples were diluted in CB, the amount of antigens coated on the plate was 2 µg/100µl. MAb 6F11 (1.19 mg/ml) was 1:1000 diluted in 1%BSA-PBST. P: pork, B: beef, L: lamb, D: deer, H: horse, C: chicken, T: turkey, Blk: blank. Each data was mean of triplicate with SD bar shown.
Figure 6a. Western blot of raw meat samples with MAb 7F6. Raw meat extracts were loaded at 4.5ug/lane. Mab 7F6 (1.541mg/ml) was 1:500 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
**Figure 6b.** Western blot of cooked meat samples with MAb 7F6. Cooked meat extracts were loaded at 3.5μg/lane. Mab 7F6 (1.541mg/ml) was 1:500 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
Figure 6c. Western blot of autoclaved meat samples with MAb 7F6. Autoclaved meat extracts were loaded at 3.5ug/lane. Mab 7F6 (1.541mg/ml) was 1:500 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
Figure 7a. Western blot of raw meat samples with MAb 6F11. Raw meat extracts were loaded at 4.5μg/lane. Mab 6F11 (0.416mg/ml) was 1:167 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
Figure 7b. Western blot of cooked meat samples with MAb 6F11. Cooked meat extracts were loaded at 3.5ug/lane. Mab 6F11 (0.416mg/ml) was 1:167 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
**Figure 7c.** Western blot of autoclaved meat samples with MAb 6F11. Autoclaved meat extracts were loaded at 3.5μg/lane. Mab 6F11 (0.416mg/ml) was 1:167 diluted in 1%BSA-TBST. P: pork, B: beef, L: lamb, H: horse, D: deer, C: chicken, T: turkey.
Figure 8. Species specificity of the optimized sandwich ELISA. MAb 7F6 (1.541 mg/ml) was used as the capture antibody; and MAb 6F11-biotin (1.36 mg/ml) was used as the detection antibody. Raw meat samples were diluted in the same volume of 1%BSA-PBST containing 10 mM EDTA, heat-treated samples were undiluted. P: pork, B: beef, L: lamb, D: deer, H: horse, C: chicken, T: turkey, Blk: blank. The absorbance values of lamb samples were shown on the top of each bar. Each data was mean of triplicate with SD bar shown.
Figure 9. Cross-reactivity with non-meat proteins by sandwich ELISA. MAb 7F6 (1.541mg/ml) was used as the capture antibody; and MAb 6F11-biotin (1.36 mg/ml) was used as the detection antibody. Soy: soy protein; albumin: egg albumin; BSA: bovine serum albumin; NFDM: non-fat dry milk; Blk: blank. All these proteins were dissolved in water at a content of 10% (10g/100ml). After heating in water bath at 100°C for 30min, PBS-NaCl was added at ratio of 1:2 (10g/20ml). The final protein content was 10g/120ml. Each data was mean of triplicate with SD bar shown.
Figure 10. Detection limits of (a) lamb-in-pork, (b) lamb-in-beef, and (c) lamb-in-chicken by optimized sandwich ELISA. Detection limit was denoted by a circle and determined as 0.5% adulteration level for cooked lamb-in-pork and lamb-in-beef, 0.25% for cooked lamb-in-chicken by Student t-test, p < 0.05. Each data point was mean of triplicate with SD bar shown.
Figure 11. Effect of heat-treatment of ovine meat on the sandwich ELISA signals. Meat samples were treated with different conditions: cooked at 100°C for 15, 30 and 60 minutes; autoclaved at 121°C for 15, 30 and 60 minutes. Blk: blank. The absorbance values were shown on the top of each bar. Each data was at least triplicate with SD bar shown.
Figure 12. Effect of frozen storage of ovine meat on the sandwich ELISA signals. Lc: lamb cooked at 100°C for 30min, La: lamb autoclaved at 121°C/1.2 bar for 30min. Frozen for 6 mon: after purchasing from supermarket, lamb meat was ground and then refrigerated at -80°C for 6 months before thawing and extracting; Fresh prepared: after purchasing from supermarket, lamb meat was ground and then heated and extracted by PBS-NaCl solution immediately. Blk: blank. The absorbance values were shown on the top of each bar. Each data was at least triplicate with SD bar shown.
REFERENCES


Huang J, Honda W. 2006. CED: a conformational epitope database. BMC Immunology 7:7


Nagaraj NS, Santhanam K. 2006. Effect of frozen temperature and storage time on calpains, cathepsins (b, b + l, h and d) and their endogenous inhibitors in goat muscles. J Food Biochem 30(2):155-73.


BIOGRAPHICAL SKETCH

I was born in Changchun, Jilin Province, the People’s Republic of China, on December 1, 1970. I attended the Affiliated High School of Northeast Teachers University where I laid a solid foundation for my future study and career. I went to Norman Bethune University of Medical Sciences in 1989, and received my degree in medicine in 1994. After graduation, I moved to Beijing, and started as a physician in the Chinese PLA general hospital. After completing two-year ward rotation in General Internal Medicine, I specialized in respiratory diseases. During that time, I met my husband, Likai Song, a cardiologist. Our daughter, Wenxin Song was born in November 1999.

In order to broaden life experience and seek new knowledge and skills, I decided to go to the United States with my husband. I came to Tallahassee, Florida in 2001, and began to prepare for graduate study. At the end of 2003, I joined Dr. Hsieh’s lab and focused on food safety research using immunological and biochemical techniques. I was accepted to the Florida State University fall 2004 master program in Food, Nutrition and Exercise Sciences. Since then, I entered a new field – food and nutrition sciences.