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FINAL REPORT

Date: March 15, 2006

Submitted to: Fats and Proteins Research Foundation, Inc.
16551 Old Colonial Road, Bloomington, IL 61704
&
Neogen Corporation, 620 Lesher Place, Lansing, MI 48912

Project Title: Development of monoclonal antibody-based immunoassay for rapid detection of sheep and deer/elk in rendered meats

Submitted By: Y-H. Peggy Hsieh, Professor

University/Location: Florida State University, Department of Nutrition, Food and Exercise Sciences, Tallahassee, FL 32306-1493

Project Starting Date: June 1, 2003

Project Completion Date: May 31, 2005 with no-cost 1-year extension

Should you have any questions, please feel free to contact Dr. Hsieh at yhsieh@mailer.fsu.edu, or 850-644-1744 (O).

Summary of Work

1. "**Sheep**" project (for FPRF)

The sheep project has been brought to the end this Spring, 2006. We have completed two fusions for the cooked sheep antigen plus two fusions for the autoclaved antigens (Objectives #1 & 2) obtaining a total of 19 final positive clones of cooked sheep and 18 clones of autoclaved sheep. After testing and screening of these hybridoma clones, there were 4 clones secreting monoclonal antibodies (Mabs) specific to cooked sheep antigen. Epitope mapping revealed that all of these four Mabs bind to the same site on the surface of the antigen molecule, thus it was impossible to select a pair of Mabs among them to construct a sandwich ELISA. To develop a sandwich ELISA, we must search two suitable Mabs binding to different sites on the antigen molecule.

After hundreds of testing and matching, we have chosen Mab 6F11 (high affinity to ovine protein but weak reaction with several mammalian species) to pair with Mab 7F6 (ruminant troponin-I specific) for construction of a sandwich ELISA based on their compatibility, high affinity to ovine protein, and the species-specificity of the assay. This sandwich assay exhibited a sheep-specific detectability in cooked product. The reaction signal decreases as the heating time and temperature increase. This ovine-specific sandwich assay has been optimized in the past few months to achieve a detection limit of 0.25% to 0.5% of cooked sheep mixed in beef, pork or chicken. The results are attached to this report.

2. "**Deer**" project (for Neogen)

Neogen Co. would like to develop an assay which is able to detect all ruminant species including deer and elk in rendered animal products. Current Reveal Ruminant Assay marketed by Neogen cannot detect elk and deer. We have completed the proposed project by conducting three fusion projects for cooked deer antigen plus two fusion projects for autoclaved deer antigen. (Objectives #1 & 2). A total of 22 final positive clones of cooked sheep and only 2 clones of autoclaved deer were obtained from these five fusion projects. As for the autoclaved sheep project, the autoclaved deer project was not quite successful receiving only cross reactive and low affinity Mabs. The reason for the unstable and non-specific clones being obtained from these fusion trials could be due to inadequate immunization period as well as the quality of meat used to extract antigen.

After exhaustive testing and screening, clone 7F6 secreting Mabs that react with all ruminant animal (bovine, ovine, cervine, elk, and buffalo) myofibril protein, troponin I was selected for further testing. We matched this Mab with all 22 newly developed Mabs and a number of Mabs previously developed in an attempt to construct a sandwich ELISA. Mab 8F10 was found to be compatible with Mab 7F6 in terms of the antigen binding site. Therefore, a sandwich ELISA was successfully developed which detects all ruminant muscle protein (troponin I) without cross reactivity with other commonly used protein additives. Effect of heat treatment of samples was also studied. The reaction signal enables a detection of the target ruminant tissue ranging from raw to excessively autoclaved meats. Detail results are provided in the attached document.

Objectives:

(1) **Preparing and Characterizing Antigens:** Characterize the species-specific region on sheep and deer/elk troponin I from sheep and deer/elk muscle, respectively and identify other major species marker proteins from sheep and deer/elk muscle

(2) **Developing Antibodies**:
a) Develop ovine-specific monoclonal antibodies (Mabs) raised against the marker protein(s)
b) Develop deer/elk-specific monoclonal antibodies (Mabs) raised against the marker protein(s)

(3) **Characterizing Antibodies**:
a) Select a pair of suitable ovine-specific Mabs for the construction of a sandwich enzyme-linked immunosorbent assay (ELISA)
b) Select a pair of suitable deer/elk-specific Mabs for the construction of a sandwich enzyme-linked immunosorbent assay (ELISA)

Brief Review of Experimental Design

Objective 1. Preparing and characterizing antigens: Characterize the species-specific region on sheep TnI from ovine muscle; and deer TnI from deer muscle and **identify and isolate other major species marker proteins from deer muscle**

Thermal-stable myofibril protein, TnI, as well as crude thermal-stable muscle proteins (TSMP) were isolated from the cooked (100^{o}C, 30 min) and autoclaved (121^{o}C, 30 min) animal (deer or sheep) muscles according to our published chromatographic methods. Proteins in these extracts were separated by SDS-PAGE and their banding patterns were compared to identify a specific antigen as the immunogen for Mab development. One antigen from each animal species were identified and isolated from each heated-treated (cooked or autoclaved) muscle extract, dialyzed to remove impurities, and then used as the immunogen.

Objective 2. Developing Antibodies: Develop a) ovine-specific Mabs raised against the sheep Tn I ; and b) deer-specific Mabs raised against the deer TnI

To develop Mabs, the general procedures described by Köhler and Milstein (1975) were followed with specific modifications used in our laboratory. The heat-treated immunogen (TnI or TSMP) was used as the immunogen but native TnI /TSMP for ELISA screening. This ensures that the selected Mabs, which recognize heat resistant epitopes appearing on both native and heat-treated antigen molecules, are capable of recognizing the antigen in both raw and heat-processed muscles. We selected only cell lines secreting IgG because of its ease of handling and good performance in immunoassay. Species specificity of the selected Mabs to sheep antigen were evaluated by indirect ELISA of muscle extracts from different species extracted by either phosphate buffered or non-buffered 0.5 NaCl solutions and with or without EDTA because pH

condition and the presence of chelator, such as EDTA would affect the selected pattern of the Mabs. The selected hybridomas were propagated in ascites fluid (Auburn University Lab) or in bioreactor (FSU Lab) for the production of large quantities of Mabs for further testing.

The reactivity of developed Mabs from above was tested against raw, cooked and autoclaved meats from all animal species using indirect ELISA. The reactivity of selected Mabs were also be tested against food protein additives including but not limited to egg albumin, gelatin, soy proteins, and milk proteins.

Objective 3. Characterizing Antibodies: **Select a) a pair of suitable ovine-specific Mabs; and a pair of deer-specific Mabs for the construction of sandwich enzyme-linked immunosorbent assays (ELISA) for sheep test and deer test, respectively.**

The feasibility of using these Mabs in indirect ELISAs for the detection of rendered muscle tissues in feedstuffs were examined by adulterating the laboratory produced sheep or deer meal into other meat meal samples. The requirement for development of a Mab-based sandwich ELISA is to select two suitable Mabs to bind to distal epitopes on the antigen molecules. After optimizing the experimental conditions, an inhibition competitive assay were used to test all the selected Mabs. An epitope map was constructed from the results. Mabs that exhibit little or no inhibition were selected for the subsequent development of a sandwich ELISA. Several previously developed Mabs, which also react with ruminant animal species were included in the testing for constructing a sandwich ELISA.

For constructing and optimizing a sandwich ELISA using the finally selected Mab, one Mab was conjugated to biotin as the detecting Mab and the other was coated on the microtiter plates as the capturing Mab. The format of reversed construction of the capturing Mab and the detecting Mab was also tested to optimize the sensitivity of the assay. Statistical analysis of the data were performed using the software SPSS. Significant difference level was established at $P<0.05$.

Results and Brief Discussion

Because of the huge volume of data obtained from this research project, results from only the final products, the sandwich assays, are presented in this report. Please see the results in the attached file. The P.I. requests that all results are kept confidential due to her patent-pending and patent application status.

Results

1) Sheep sandwich ELISA:

Purified Mab 7F6 (!:10,000 diluted in phosphate buffered saline, PBS) was used as the capture antibody and biotinylated Mab6F11 was used as the detection antibody (1.36 mg/ml, 1:1000 diluted in 1%BSA-PBST) for the sandwich ELISA. Meat was extracted in PBS containing 0.5 M NaCl. Cooked meat was heated at 100°C for 30min and autoclaved meat was autoclaved at 121°C for 30min. The species-specificity of the Mab 6F11 (Figure 2) and its antigenic components (Figures 7-9) in the raw, cooked and autoclaved meat extracts are presented in this section. The species-specificity of Mab 7F6 and its antigenic components in meat extracts are shown in the section of "Ruminant sandwich ELISA." Each data was obtained from at least tree samples. Standard deviation bars are shown in the diagrams.

(a) Performance of the sandwich ELISA using Mab 6F10 and Mab 7F6

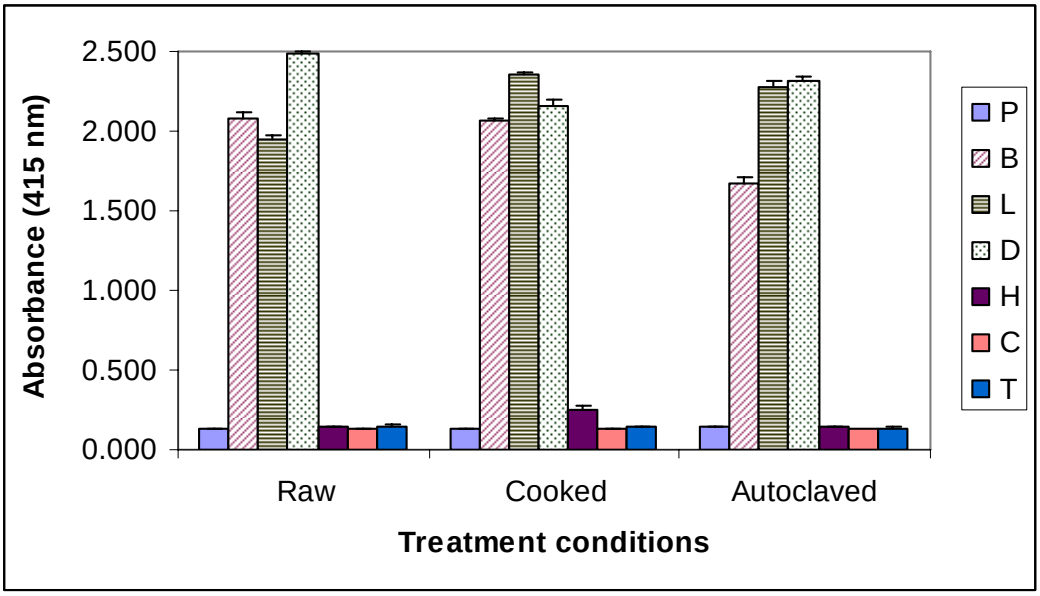


Figure 1. Species specificity of Mab 7F6 by indirect ELISA.
Pork (P), beef (B), lamb (L), deer (D), horse (H), chicken (C), and turkey (T).

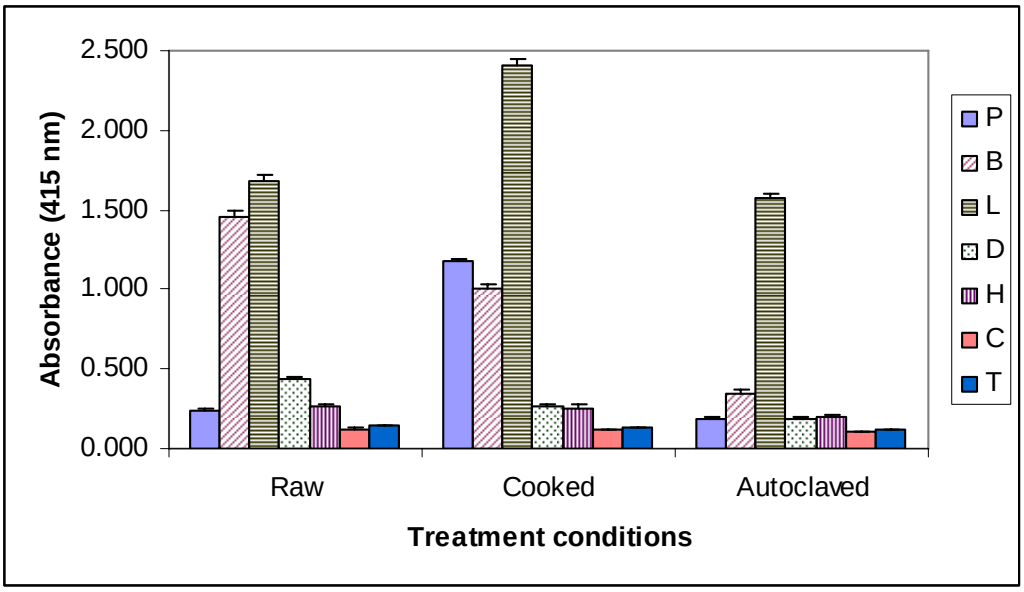


Figure 2. Species specificity of Mab 6F11 by indirect ELISA.
Pork (P), beef (B), lamb (L), deer (D), horse (H), chicken (C), and turkey (T).

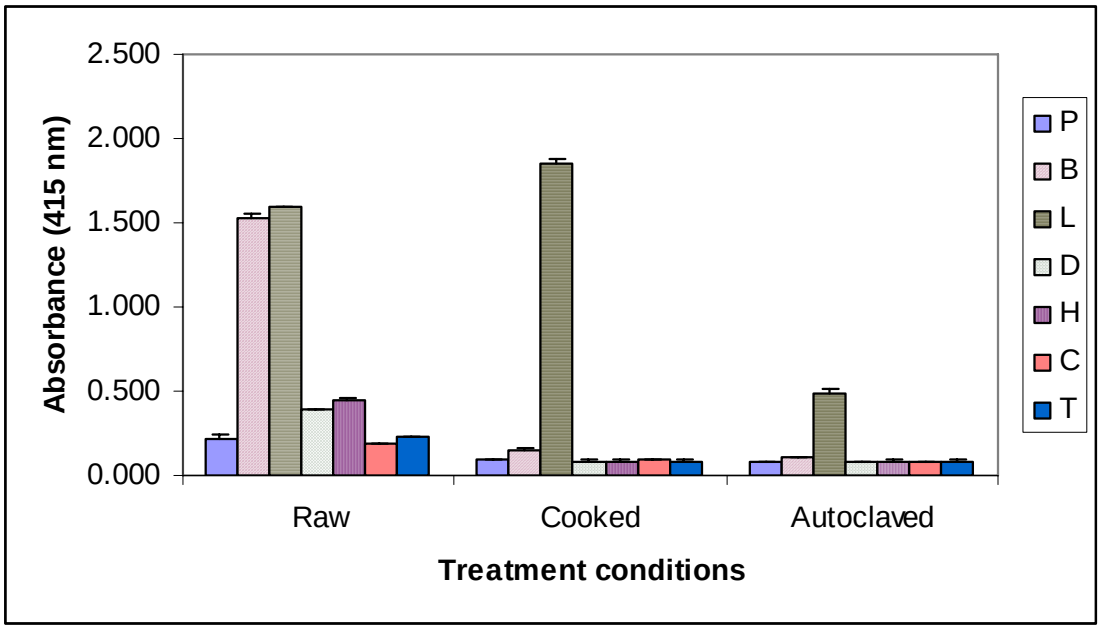


Figure 3. Species specificity of the sandwich ELISA. Pork (P), beef (B), lamb (L), deer (D), horse (H), chicken (C), and turkey (T).

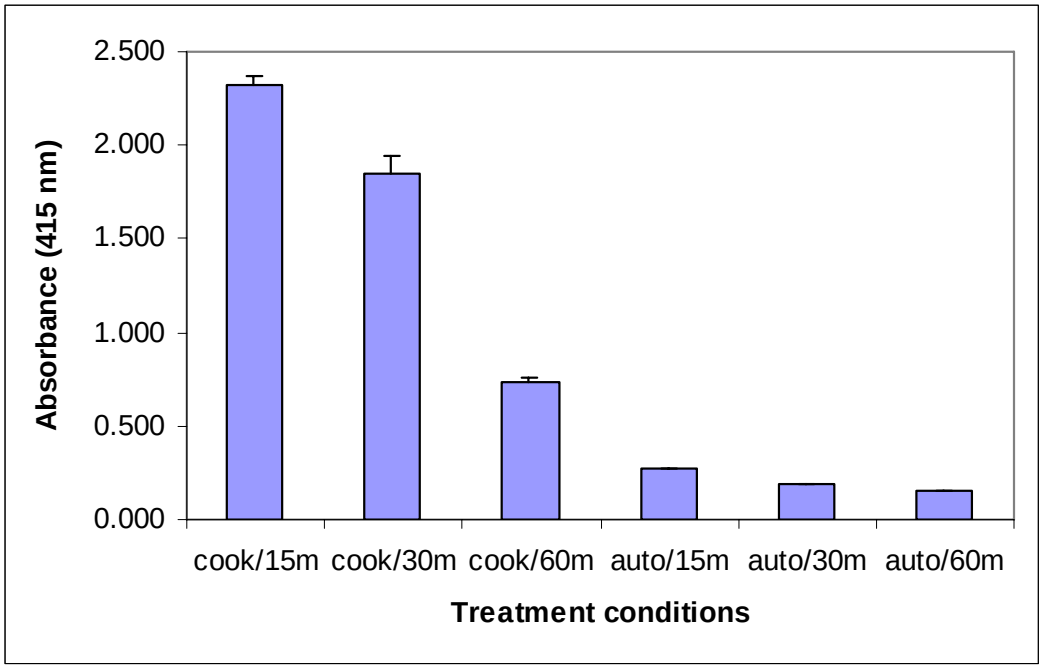
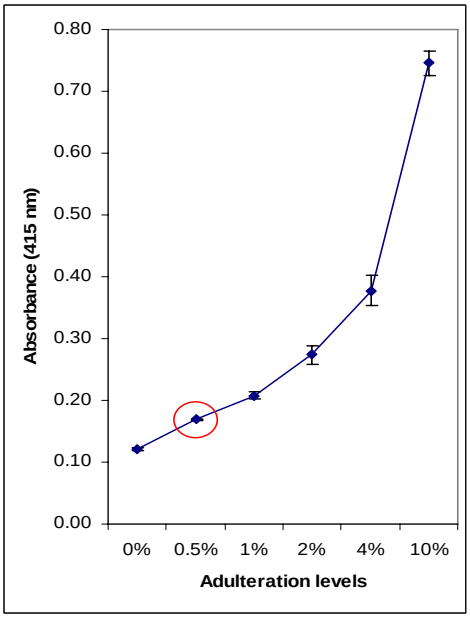


Figure 4. Effect of heat treatment of sheep meat samples on reactivity of the sandwich ELISA. Meat samples were treated in deferent conditions: cooked at100°C for 15, 30 and 60 minutes; autoclaved at 121°C for 15, 30 and 60 minutes.



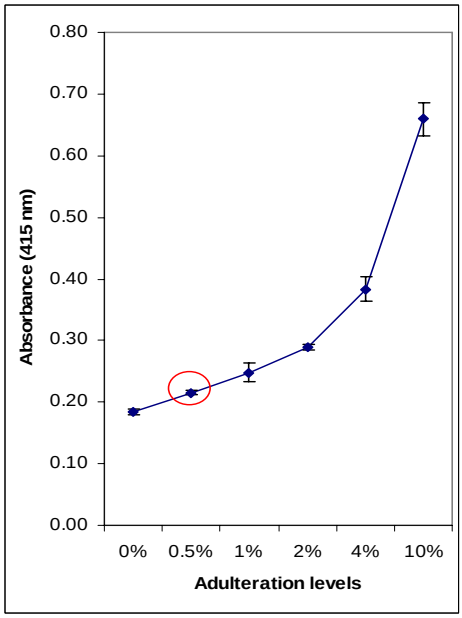


Figure 5a. Detection limit of the sandwich ELIISA for cooked sheep adulterated in pork. Detection limit was 0.5% by student t-Test, $p < 0.05$.
Figure 5b. Detection limit of the sandwich ELISA for cooked sheep adulterated in beef. Detection limit was 0.5% by student t-Test, $p < 0.05$

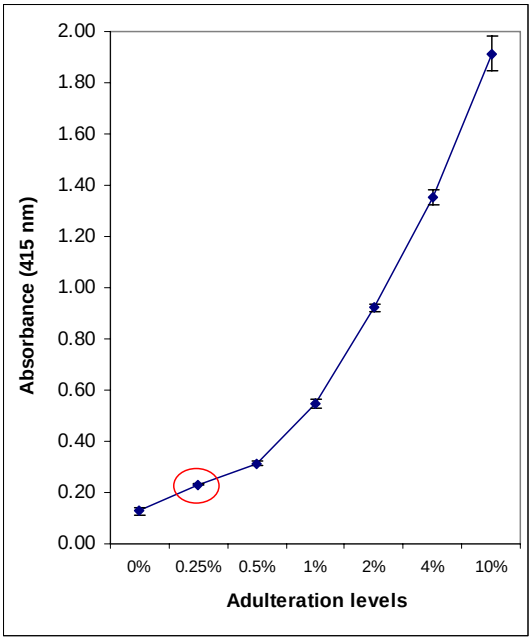


Figure 5c. Detection limit of the sandwich ELISA for cooked sheep adulterated in chicken. Detection limit was 0.25% by student t-Test, $p<0.05$.

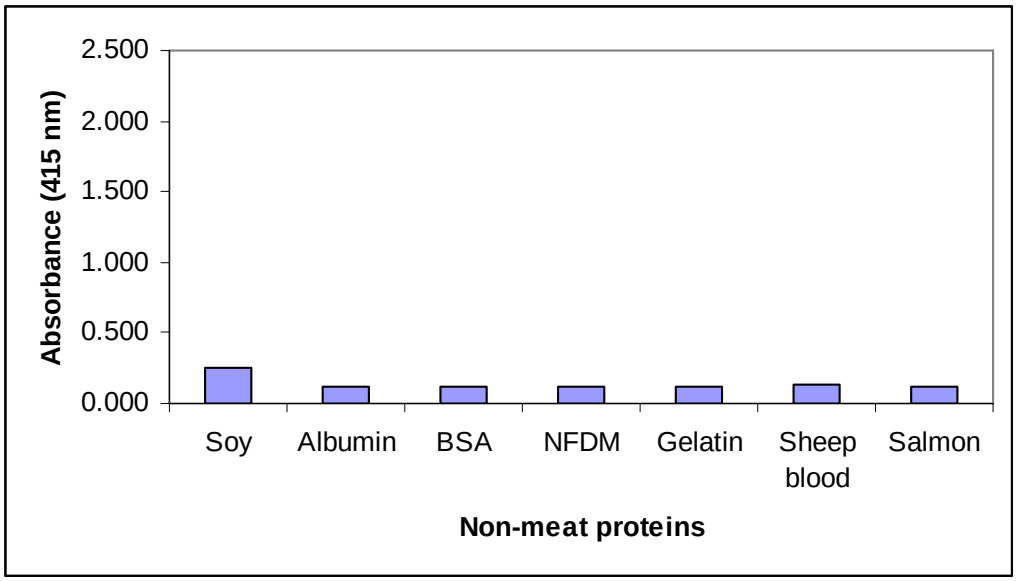


Figure 6**.** Cross-reactivity of cooked sheep sandwich ELISA. Soy: soy protein; albumin: egg albumin; BSA: bovine serum albumin; NFDM: non-fat dry milk. All these proteins were 10% (10g/100ml) extracted in water, after cooking at 100°C for 30min, PBS-NaCl was added at ratio of 1:2(10g/20ml), the final concentration was 10g/120ml.

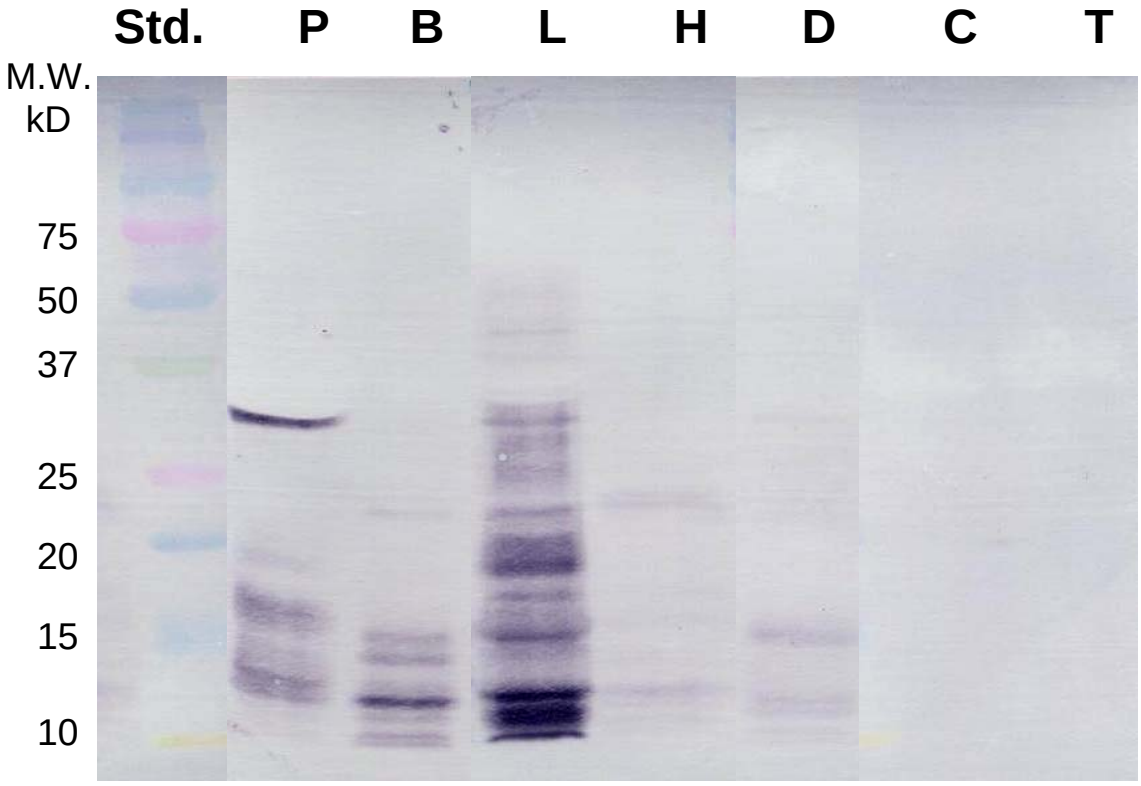


Figure 7. Western blot showing antigenic protein bands in raw meat extracts probed with Mab 6F11. Pork (P), beef (B), lamb (L), deer (D), horse (H), chicken (C), and turkey (T).

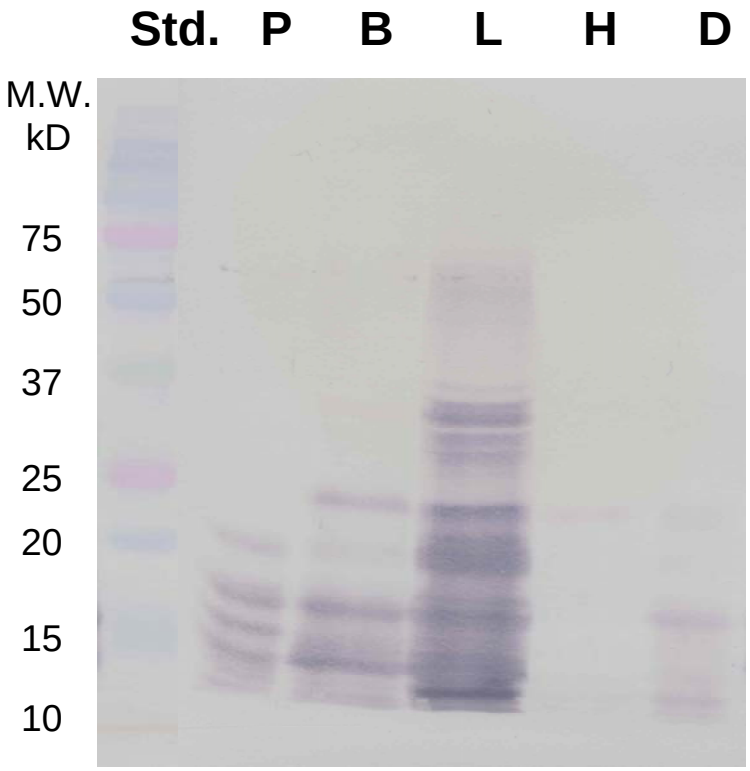


Figure 8. Western blot showing antigenic protein bands in cooked meat extracts probed with Mab 6F11. Pork (P), beef (B), lamb (L), deer (D), and horse (H).

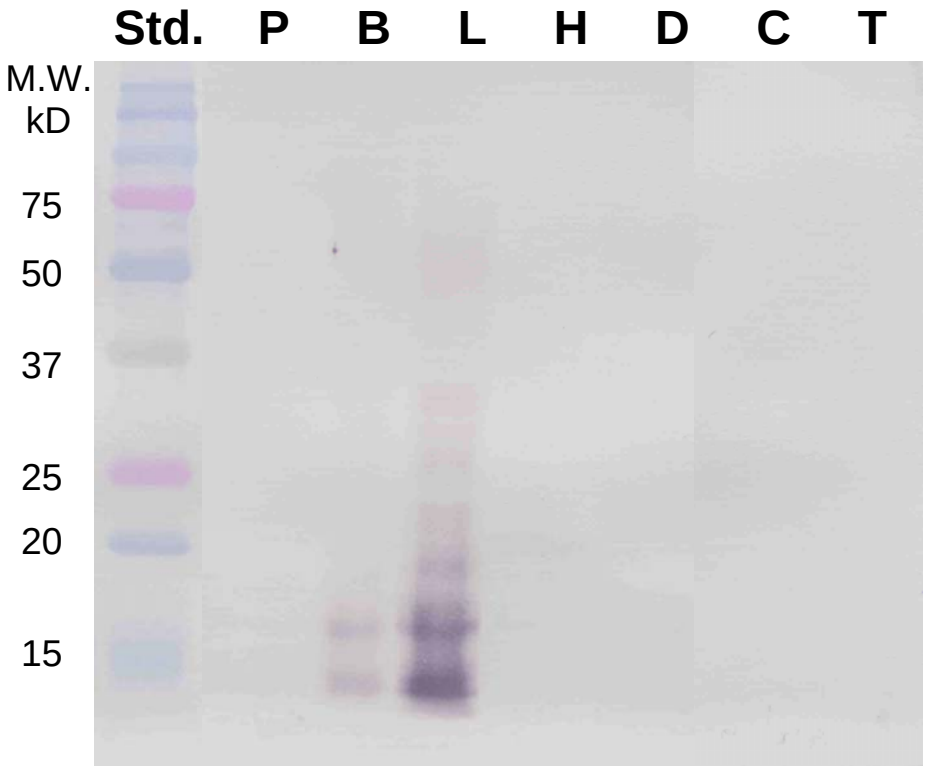


Figure 9. Western blot showing antigenic protein bands in autoclaved meat extracts probed with Mab 6F11. Pork (P), beef (B), lamb (L), deer (D), horse (H), chicken (C), and turkey (T).

2) Ruminant (Deer Project) sandwich ELISA:

The ruminat sandwich ELISA using Mab 8F10 as the capture antibody and biotinylated Mab 7F6 as the detecting antibody is an improved immunoassay which detects muscle protein (troponin I) from all ruminant animals including cattle, buffalo, elk, deer, sheep and goat. The assay can be used to analyze raw, cooked and autoclaved samples without cross reactivity with common protein additives, such as gelatin, milk proteins, soy proteins, blood proteins and fish proteins. However, the assay gave a weak positive signal to raw egg albumin. The assay signal will be diminished if the samples are treated up to132^{o}C for 2 hours. Prolonged autoclaving might have destroyed epitope on the target antigen.

a) Characterization of Mab 7F6

Following figures demonstrate the species specificity of Mab 7F6 (Figures 1-2), heat stability of the epitopes (Figure 3), and the antigenic components in beef protein extracts using indirect ELISA with Mab 7F6 (Figures 4-6).

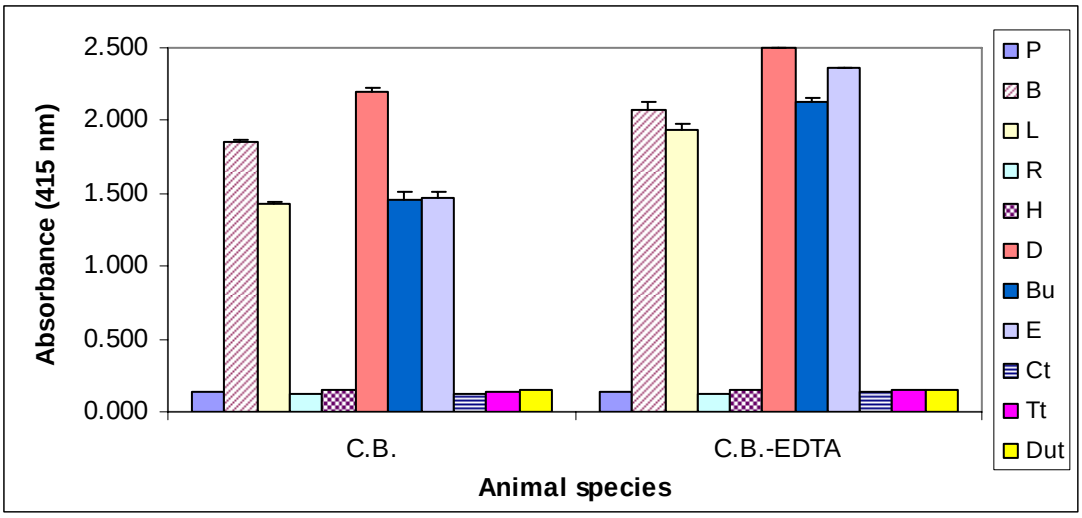


Figure1. Species specificity of MAb 7F6 to raw meat samples. Raw samples were diluted (2 µg/100µl) in carbonate buffer with or without 10mM EDTA. 7F6 (1.541mg/ml, 1:3000 diluted in 1%BSA-PBST).

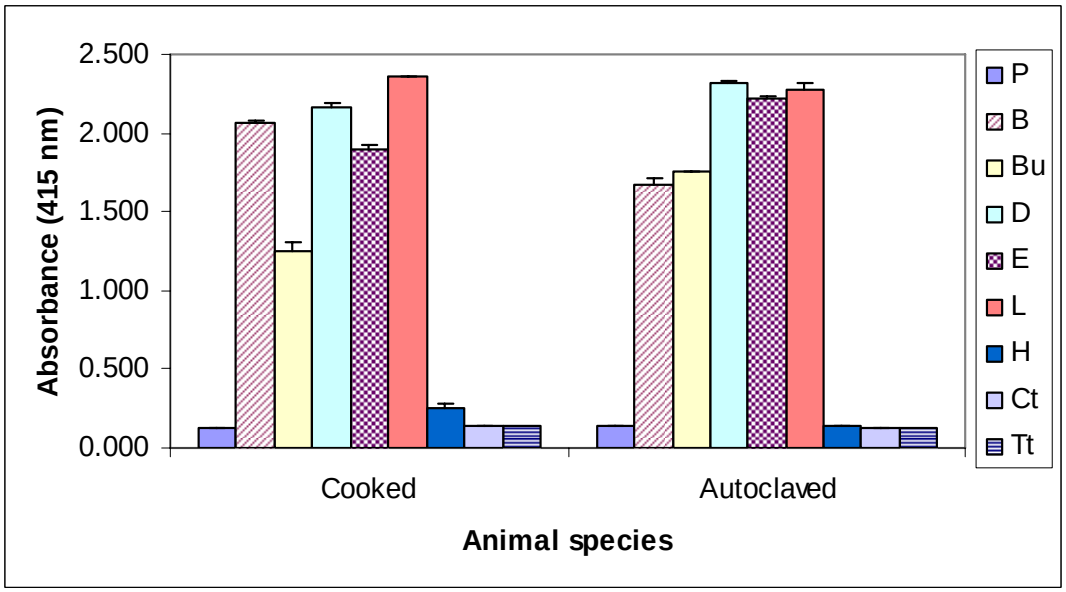


Figure 2. Species specificity of MAb 7F6 to cooked and autoclaved meat samples. Meat samples were diluted (2 µg/100µl) in carbonate buffer without EDTA.

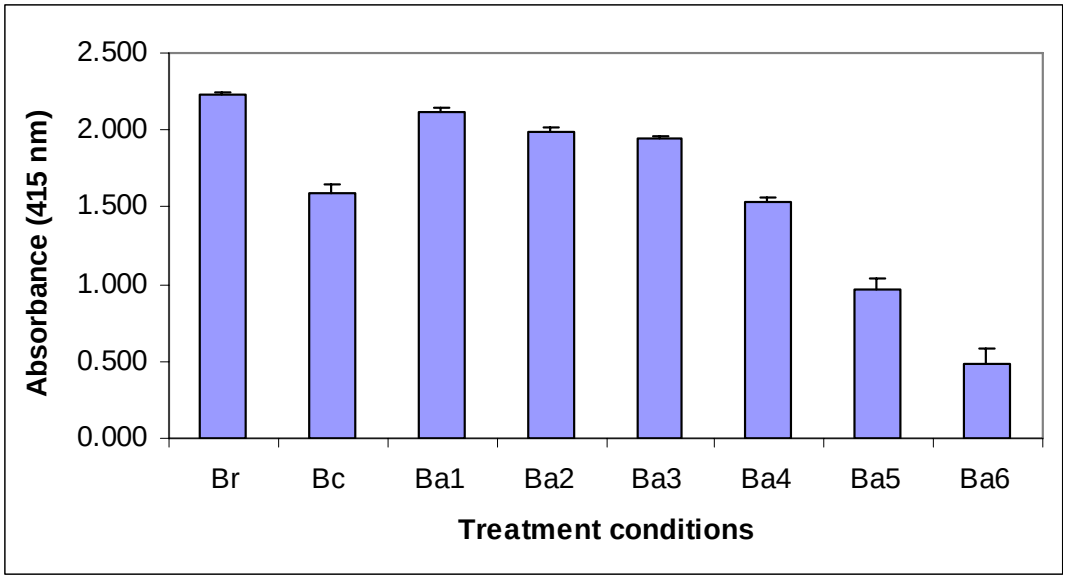


Figure 3. Effect of heat-treatment of beef samples on the indirect ELISA signals probed with Mab 7F6. Raw meat samples were diluted (2 µg/100µl) in carbonate buffer with EDTA, cooked and autoclaved meat samples were diluted (2 µg/100µl) in carbonate buffer without EDTA. Br: untreated, Bc: 100°C/30min, Ba_1: 121°C/30min, Ba_2: 128°C/30min, Ba_3: 132°C/30min, Ba_4: 132°C/60min, Ba_5: 132°C/90min, Ba_6: 132°C/120min.

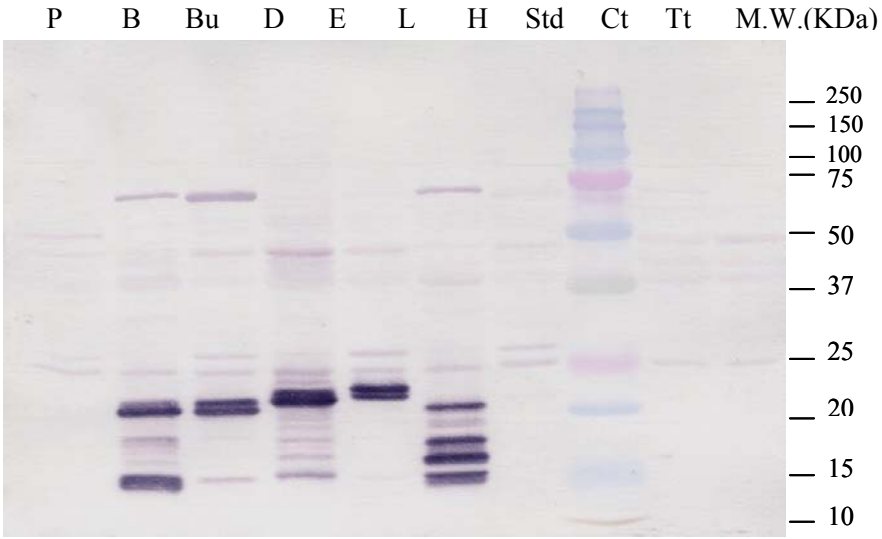


Figure 4. Representative Western blot indicating specificity of MAb 7F6 to raw meats from different species. Pork (P), beef (B), buffalo (Bu), deer (D), elk (E), lamb (L), horse (H), molecular weight standard (Std), chicken thigh (Ct), and turkey thigh (Tt)

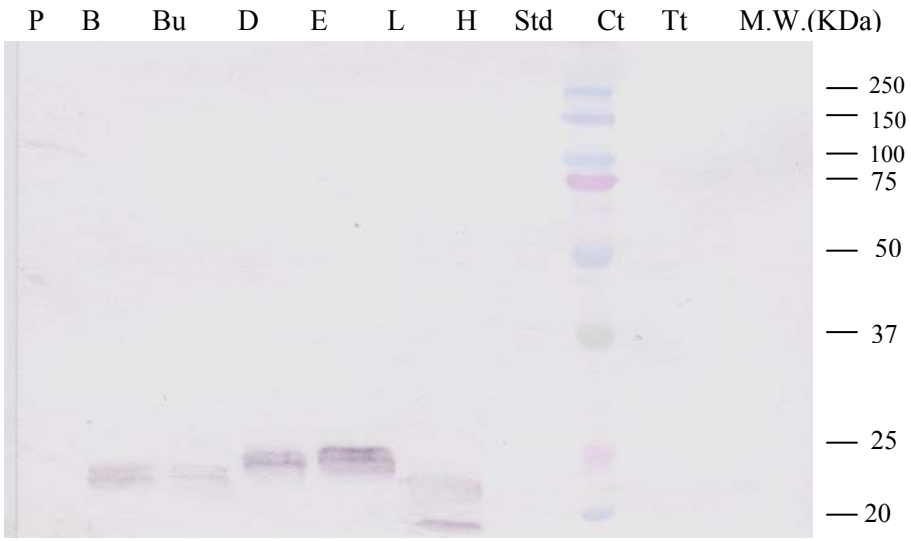


Figure 5. Representative Western blot indicating specificity of MAb 7F6 to cooked meats from different species. Pork (P), beef (B), buffalo (Bu), deer (D), elk (E), lamb (L), horse (H), molecular weight standard (Std), chicken thigh (Ct), and turkey thigh (Tt)

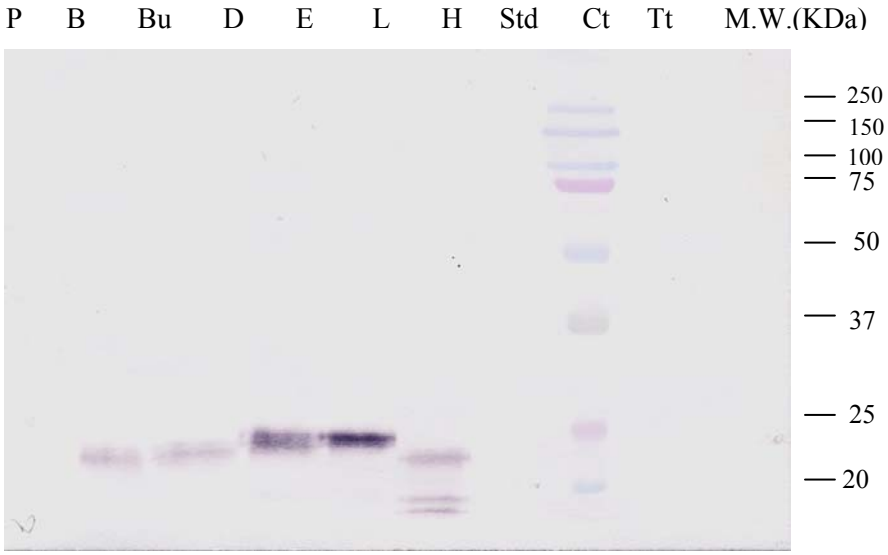


Figure 6. Representative Western blot indicating specificity of MAb 7F6 to autoclaved meats from different species. Pork (P), beef (B), buffalo (Bu), deer (D), elk (E), lamb (L), horse (H), molecular weight standard (Std), chicken thigh (Ct), and turkey thigh (Tt)

(b) Performance of the sandwich ELISA (Mab 8F10 with biotinylated Mab 7F6)

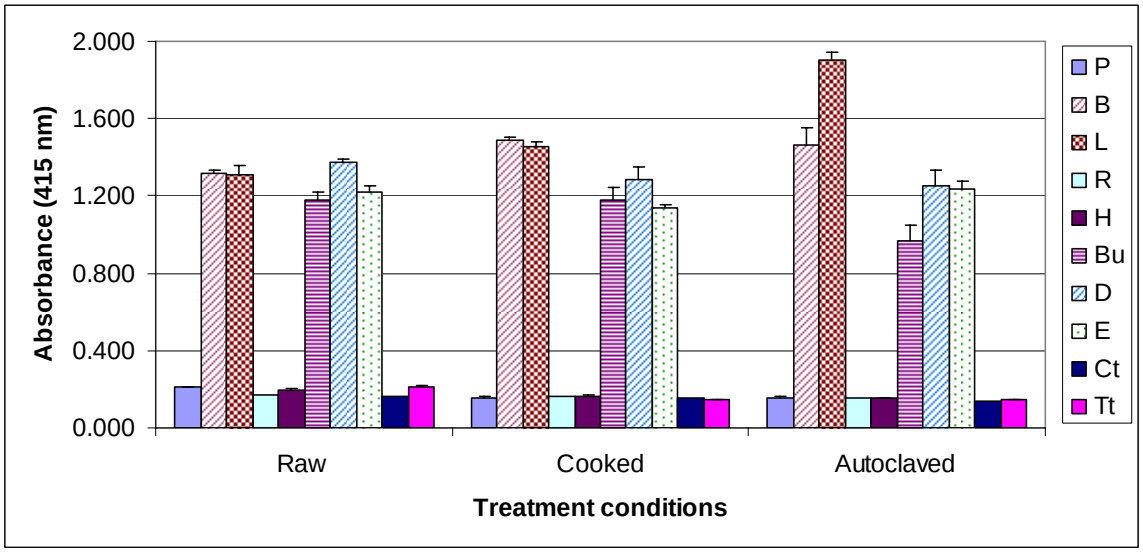


Figure 7. The species specificity determined by MAbs 8F10 and 7F6-biotin based sandwich ELISA. Raw meat samples were 1:1 diluted in PBS buffer containing 1% BSA, 0.05% Tween-20 and 10 mM EDTA. Cooked and autoclaved meat samples were undiluted. 8F10 (1.64mg/ml, 1:1000), 7F6-biotin (0.84mg/ml, 1:500).

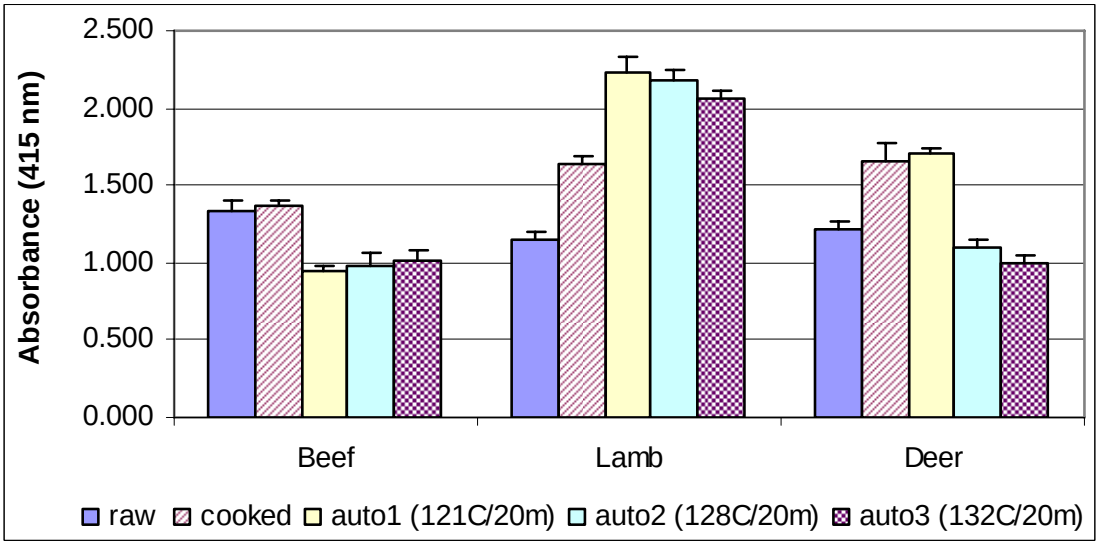


Figure 8. Effect of heat-treatment on ruminant sandwich ELISA. Raw meat samples were 1:1 diluted in 1% BSA-PBST containing 10 mM EDTA. Cooked and autoclaved meat samples were undiluted. Raw: untreated, cooked: 100°C/20min, auto1: 121°C/20min, auto2: 128°C/20min, auto3: 132°C/20min. 8F10 (1.64mg/ml, 1:3000), 7F6-biotin (0.84mg/ml, 1:1000).

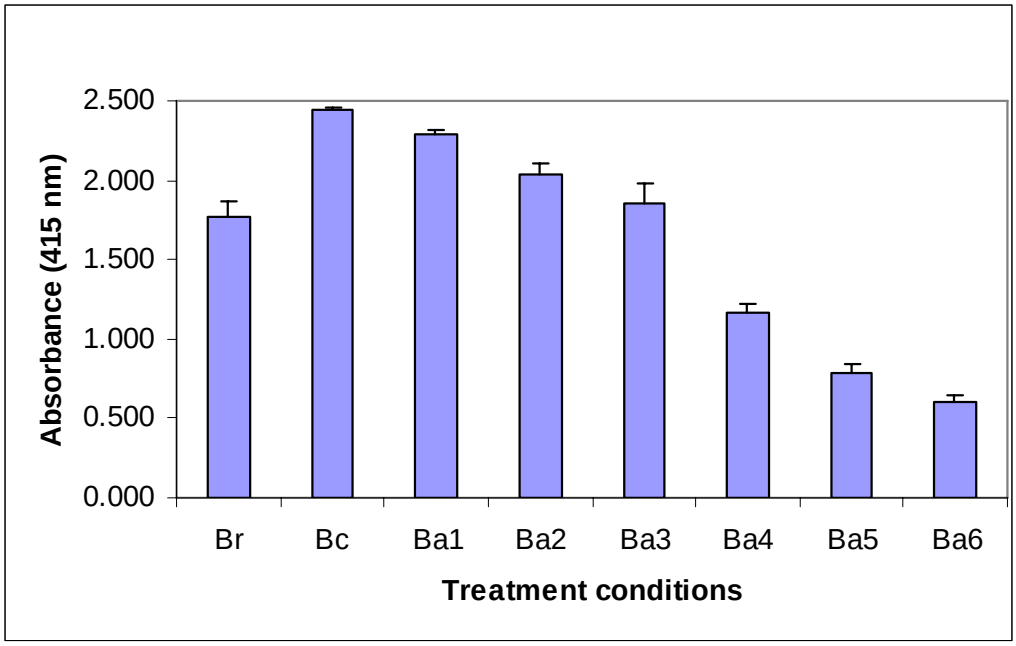


Figure 9. Effect of heat-treatment on ruminant sandwich ELISA. Raw meat samples were 1:1 diluted in 1% BSA-PBST containing 10 mM EDTA. Cooked and autoclaved meat samples were undiluted. All samples were beef meat treated under different conditions. Br: untreated, Bc: 100°C/30min, Ba_1: 121°C/30min, Ba_2: 128°C/30min, Ba_3: 132°C/30min, Ba_4: 132°C/60min, Ba_5: 132°C/90min, Ba_6: 132°C/120min. 8F10 (1.64mg/ml, 1:1000), 7F6-biotin (0.84mg/ml, 1:500).

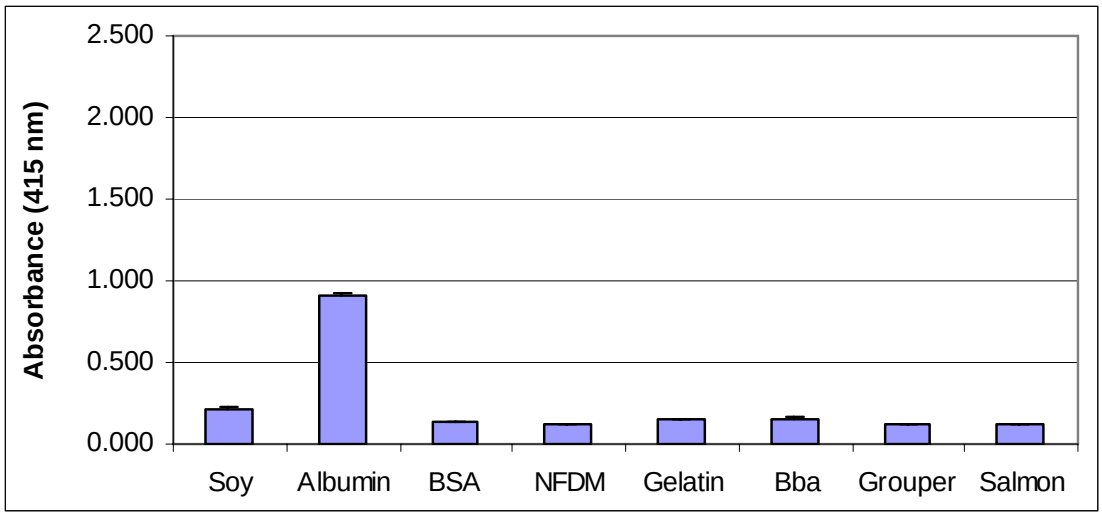


Figure 10. Cross-reactivity of ruminant sandwich ELISA to other raw common proteins. Soy: soy protein concentrate, Albumin: egg albumin, BSA: bovine serum albumin, NFDM: non-fat dry milk, Bba: beef blood autoclaved at 121°C/30min, Grouper: autoclaved at 121°C/30min, Salmon: Norwegian Salmon autoclaved at 121°C/30min. Soy, Albumin, BSA, NFDM, Gelatin were dissolved in 0.5M NaCl solution (5% wt/vl). 8F10 (1.64mg/ml, 1:3000), 7F6-biotin (0.84mg/ml, 1:1000).

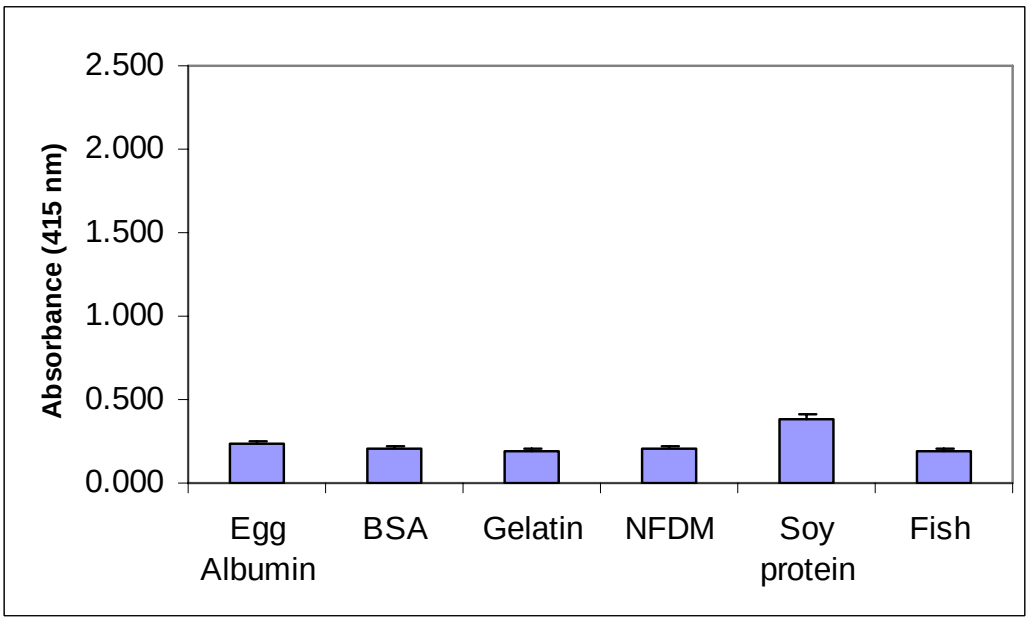


Figure 11. Cross-reactivity of ruminant sandwich ELISA to other cooked common proteins. BSA: bovine serum albumin, NFDM: non-fat dry milk, fish: autoclaved at 121°C/30min. Egg Albumin, BSA, Gelatin, NFDM, and Soy protein were dissolved in 0.5M NaCl solution (10% wt/vl), after cooking at 100°C/30min, addition of 0.5M NaCl solution was added (1:2, wt/vl). 8F10 (1.64mg/ml, 1:3000), 7F6-biotin (0.84mg/ml, 1:1000).