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Monoclonal Antibody–Based Sandwich Enzyme-Linked Immunosorbent Assay for Sensitive Detection of Prohibited Ruminant Proteins in Feedstuffs

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ABSTRACT

Regulations aimed to control the epidemic of bovine spongiform encephalopathy have banned the use of certain animal products, i.e., ruminant meat and bone meals, in ruminant animal feeds. A sensitive enzyme-linked immunosorbent assay has been developed to detect prohibited bovine and ovine muscles in feedstuffs. The assay utilizes a pair of monoclonal antibodies (MAbs) against skeletal troponin I (TnI). MAb 5G9, specific to bovine and ovine TnI, was used as the capture antibody and the biotin-conjugated MAb 2G3, reacting to all heterologous TnI, was used as the detection antibody. Quantitative procedures were applied to samples containing 5, 0.5, and 0.05% (wt/wt) of heat-treated (132°C/2 bar, 2 h) bovine and ovine meat meals in three different feeds, coexisting with porcine, chicken, or turkey meat meal. The presence of these nonprohibited species did not affect the detection of bovine and ovine meat meals in the feed samples ($P > 0.05$). Quantitative determinations of extractable bovine and ovine TnI, with a detection limit of 5.0 and 4.0 ng/ml, respectively, were achieved when the matching feed matrixes were used in the calibration curves. This new assay provides a rapid and reliable way to detect animal protein products containing a trace amount of bovine or ovine muscle tissue in feedstuffs.

Bovine spongiform encephalopathy (BSE), also known as mad cow disease, has had a significant impact on the beef industry. Issues concerning transmission of the disease and potential health hazards associated with the BSE agent contaminating beef products continue to challenge regulatory authorities. Regulations designed to control the epidemic of BSE have banned the use of certain animal products. The use of mammalian meat and bone meals to feed any farmed livestock, including fish and horses, has been prohibited in the United Kingdom *(1).* In addition, a European Union–wide feed control program has also introduced a ban on the feeding of processed animal protein to animals that are kept, fattened, or bred for the production of food under the Processed Animal Protein Regulations *(3).* Although there have been no reported cases in the United States to date, in order to prevent the establishment and spread of BSE, the Food and Drug Administration has banned the use of proteins derived from mammalian tissue (e.g., meat and bone meals from cattle and sheep) to feed ruminant animals *(2).* According to this regulation, certain products, such as blood and milk products and pure protein products from porcine and equine sources, are exempt from this restriction.

Analytical methods capable of determining the constituents of animal origin in feedstuffs are vital for successful regulatory enforcement. Methods based on polymerase chain reaction amplification of target DNA sequences are species-specific and sensitive in nature, but are generally not able to distinguish between different tissues of the same species *(14).* The official enzyme immunoassay method for analyzing mammalian proteins of rendered animal material in the United Kingdom utilizes antisera raised against heat-stable proteins *(4).* This method detects most mammalian proteins without discriminating between prohibited and permitted animal proteins. Therefore, there is a need to improve existing methods or develop new methods for the detection of prohibited animal proteins with regard to species and tissue origins.

Muscle tissue is present in substantial amounts in rendered animal products such as meat and bone meal. Immunoassays based on the detection of muscle components of a particular species would offer a way to differentiate the animal proteins of prohibited species from those that are allowed. Our previous efforts have demonstrated the applications of troponin I (TnI), a regulatory myofibrillar protein, as a thermostable marker protein for species identification in severely heated meats *(8, 9).* Monoclonal antibodies (MAbs) specific to porcine muscle have also been successfully developed for the detection of pork in heat-processed meat products *(10).* The enzyme-linked immunosorbent assay (ELISA) specifically detects porcine skeletal muscle without cross-reaction to blood, milk, and gelatin *(7).* In light of these studies, methods based on the detection of TnI would provide a muscle-specific assay to distinguish proteins of meat and bone meals from those of blood and milk and to differentiate species of prohibited

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animals from those that are permitted. Therefore, our objectives were (1) to produce MAbs with distinct species specificity to the thermostable marker protein—TnI, and (2) to develop and evaluate a MAb-based sandwich ELISA for the detection of prohibited animal proteins in feedstuffs.

MATERIALS AND METHODS

Preparation of muscle TnI. Muscle tissues from leg portions of individual species were used for the preparation of TnI and meat meals. Fresh pork ham, beef round, leg of lamb, and turkey and chicken thighs were obtained from the Auburn University Meat Laboratory. Horse and deer round were received from the Auburn University College of Veterinary Medicine. All meats were at least 24-h postmortem and without previous freezing and processing. Skeletal muscles from the leg portions were exercised and external fat and connective tissues were trimmed off. The muscle tissues were finely ground and thoroughly mixed. All utensils were completely cleaned and dried before muscles of different species were processed. The mixtures were then divided into aliquots of about 100 g each and stored at −80°C until use.

Bovine troponin (Tn) complex was extracted from the skeletal muscle using 0.8 M LiCl *(12).* Individual components of Tn (TnI, TnC, and TnT) were fractionated by diethylaminoethyl cellulose (DE52; Whatman, Fairfield, N.J.) followed by carboxymethyl cellulose (CM52; Whatman) chromatography *(15).* TnIs of other species (porcine, ovine, deer, chicken, and turkey) were purified individually from skeletal muscle by affinity chromatography *(16),* utilizing the calcium-dependent binding capability of bovine TnC to various TnIs. Purified TnIs were dialyzed in 0.01 M sodium phosphate buffer containing 0.5 M NaCl and stored at −20°C. Protein concentrations of the preparations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, Calif.) using bovine serum albumin (BSA) as the standard.

Production of MAbs. Bovine TnI (1.0 mg/ml) in phosphate buffered saline (PBS, 0.01 M sodium phosphate and 0.15 M sodium chloride, pH 7.2) containing 0.3% of sodium dodecyl sulfate was heated in boiling water for 30 min. Four BALB/c mice (7 to 10 weeks old) were immunized either subcutaneously or intraperitoneally with 150 μg of the heat-treated TnI mixed 1:1 (vol/vol) with Freund's complete adjuvant followed by two booster injections at 4-week intervals with 100 μg per mouse of TnI mixed 1:1 (vol/vol) with Freund's incomplete adjuvant. Test sera were collected by tail bleeding 10 days after each injection; the titer of the sera was then determined by indirect ELISA.

The mouse exhibiting the highest serum titer to bovine TnI then received a final boost of 100 μg of the antigen in PBS 4 days before the fusion. Spleen cells from the selected mouse were fused with the myeloma cell line (P3×63.Ag8.653., ATCC CRL 1580) at a ratio of 5:1 in the presence of polyethylene glycol (molecular weight, 4,000). The cells were subsequently diluted to an appropriate density and cultured in hypoxanthine-aminopterin-thymidine medium. The medium was changed twice to remove residual antibodies before the initial screening against bovine TnI using indirect ELISA. For secondary selection, the positive cells from the initial screening were transferred to larger wells and cultured for 3 more days before testing for reactivity with TnIs from other species. In this way, hybridomas with distinct reaction patterns to heterologous TnI were selected. The cell lines were cloned at least twice by a limiting dilution method and subsequently maintained in liquid nitrogen.

Ascites fluid containing MAbs were obtained from Pristane primed mice 10 to 14 days after intraperitoneal injection of the hybridoma cells. Antibodies were separated from the ascites fluid using a Protein A Cartridge with MAPS II buffer (Bio-Rad). The purified antibodies were dialyzed against PBS overnight at 4°C with several changes of dialysis buffer. The concentration of IgG in the final preparation was determined by UV absorption at 280 nm. The biotin-conjugated MAbs were prepared using biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma, St. Louis, Mo.) following a standard protocol *(13).* The purified IgG and biotin conjugates were stored at −20°C with addition of 50% glycerol and 0.05% of thimerosal.

Immunoassay procedures. For hybridoma screening and titration of MAbs, polyvinylchloride microtiter plates (Costar, Cambridge, Mass.) were coated with TnI (0.2 μg/ml) in carbonate buffer (pH 9.6) and blocked with 1% of BSA in PBS. Either culture supernatant or series diluted MAbs in antibody buffer (1% BSA in PBS containing 0.05% of Tween 20) were added to the wells and incubated for 1 h at 37°C, followed by the addition and incubation of goat anti-mouse IgG peroxidase conjugate (Bio-Rad), 1:3,000 diluted in antibody buffer. Plates were washed three times with PBS containing 0.05% of Tween 20 between each step. The bound enzyme activity was revealed by the addition of an enzyme substrate (22 mg of 2,2′-azino-di-[3-ethyl-benzothiazoline-6-sulfonic acid] and 15 μl of 30% hydrogen peroxide in 100 ml of 0.1 M phosphate-citrate buffer, pH 4.0). The reaction was stopped after 10 min by the addition of 0.2 M citric acid. Color developments were evaluated spectrophotometrically at 415 nm using a microplate reader (Model 450; Bio-Rad).

For sandwich ELISA, microplates were coated with MAb 5G9 (5 μg/ml in PBS) and blocked with 1% of BSA in PBS. Sample extracts diluted with the same volume of assay buffer (1% BSA in PBS containing 0.05% of Tween 20 and 10 mM EDTA) were added to the wells and incubated at 37°C for 1 h. Biotin-conjugated MAb (0.1 μg/ml in antibody buffer) was added to the wells and incubated for another hour at 37°C, followed by the addition and incubation of streptoavidin-peroxidase conjugate (Sigma), 0.2 μg/ml in antibody buffer. The color development procedures were the same as described above.

Preparation of meat meals and feed samples. Meat meals from individual species were produced in our laboratory. Ground muscles of beef, pork, lamb, horse, deer, chicken, and turkey were placed in beakers covered loosely with aluminum foil. The muscles were autoclaved at 132°C/2 bar for 2 h; the meat slurries were then dried on sheets of aluminum foil in a convection oven at 80°C for 24 h. Dry weights of the final products ranged from 23 to 27% of the original material. Dried meats were crumbled into fine granules with a food processor and then individually packed in sealed plastic bags and stored in a refrigerator.

Three commercial feeds containing no animal proteins were used as the matrices for adulteration of meat meals. These animal feeds were feed A—Purina Natural Beef Builder Coarse (Purina Mills Inc., St. Louis, Mo.), containing processed grain by-product, roughage products, grain products, plant protein products, and molasses products with various vitamins and mineral supplements; feed B—Chicken Scratch Feed (Southern States Cooperative Inc., Richmond, Va.), containing various grain products; and feed C—Soy Best (Grain States Soya Inc., West Point, Nebr.), containing high bypass soybean meal.

Feed samples were first ground into powder using a food processor, then a predetermined amount of meat meal was added on a dry-weight basis. For example, to produce a sample of feed A containing 10% of porcine and 5% of bovine, meat meals of porcine (10 g) and bovine (5 g) were added to 85 g of feed A. A total of 54 ($3 \times 3 \times 2 \times 3$) adulterated samples were formulated based on the three animal feeds to contain 5, 0.5, and 0.05% of

TABLE 1. *Species specificity of anti-TnI monoclonal antibodies determined by indirect ELISA and immunoblot*

MAb	Class	Species specificity[a]
1B2	IgG	Bovine, ovine
3E12	IgG	Bovine, ovine, deer
5G9	IgG	Bovine, ovine

[a] MAbs were tested against TnI from porcine, bovine, ovine, equine, deer, chicken, and turkey using indirect ELISA and immunoblot, as described previously *(11)*.

either bovine or ovine meat meals, along with 10% of the porcine, chicken, or turkey meat meals. The adulterated samples were mixed with 1:6 (wt/vol) of 0.5 M NaCl and autoclaved at 121°C for 20 min. After autoclaving, the samples were homogenized using a Polytron homogenizer and centrifuged at 15,000 × *g* for 30 min. The supernatant was filtered through a Whatman #1 filter paper and the filtrates analyzed by the sandwich ELISA. Differences of means among the feed formulations were compared using the general linear model of the SPSS (10.0 for Windows) statistics program.

RESULTS

Production and characterization of MAbs. Three hybridoma cell lines (1B2, 3E12, and 5G9) secrete MAbs with distinct reactivity to heterologous TnI were selected after screening the original fusion plates. The cell lines were cloned and MAbs were produced in mice as ascites fluid, from which IgG was purified for the development of immunoassay. The species-specificity of the MAbs was confirmed by indirect ELISA and immunoblot. As summarized in Table 1, MAbs 1B2 and 5G9 recognized TnI of bovine and ovine only, while MAb 3E12 reacted to TnI of ruminant species (bovine, ovine, and deer). All MAbs showed no reactivity to TnI from porcine, equine, chicken, and turkey.

MAb 5G9 consistently showed a higher titer toward bovine TnI than MAbs 1B2 and 3E12 (Fig. 1). Similar trends were observed when the same MAbs were titrated against ovine TnI (data not shown). These titration curves were used as an indication of relative affinity of the MAbs toward TnI *(11),* suggesting a higher affinity for the MAb 5G9 than the other two MAbs and potentially a higher sensitivity in the final assay format. Therefore, MAb 5G9 was selected for the development of the sandwich ELISA. There were also discrepancies in the relative affinity toward heterologous TnI within the same MAb. All three MAbs exhibited a higher titer to ovine TnI than to bovine TnI, indicating a higher affinity of MAbs toward ovine TnI despite that fact that the bovine TnI was the immunogen used in the development of the MAbs.

Development of sandwich ELISA. A sandwich ELISA was constructed for the detection of TnI in bovine and ovine meat meals. The initial combinations tested, where MAb 5G9 was used with MAb 1B2 or 3E12 to form a pair of capture and detection antibodies, was unsuccessful, probably due to steric-hindrance resulting from the overlapping or adjacent epitopes of these MAbs that prevents them from binding collaterally to TnI. However, a

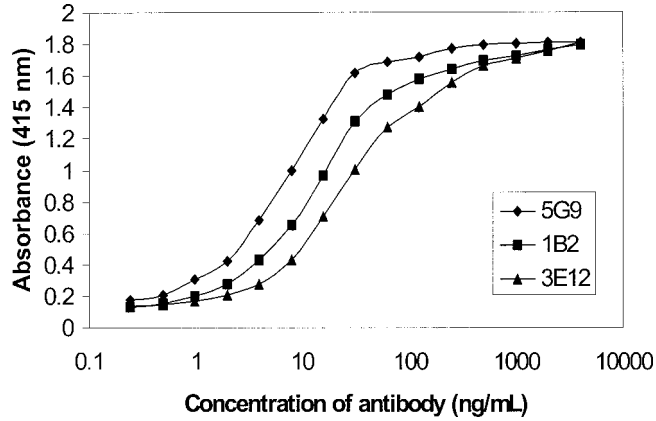

FIGURE 1. *Titration curves of purified anti-TnI MAbs against bovine TnI by indirect ELISA.*

previously developed antibody, MAb 2G3 *(11),* which displays a broad reactivity to TnI of different species (mammalians and fishes), was subsequently utilized successfully as the detection antibody. MAb 2G3 apparently recognizes an epitope distinct from that of the capture MAb 5G9 coated on the well surface of the microplate. This configuration allows simultaneous binding of MAb 5G9 and biotin-conjugated MAb 2G3 to TnI, which is subsequently detected by streptoavidin-peroxidase conjugate and revealed by color development with the enzyme substrate.

The assay conditions, including the concentrations of capture and detecting antibodies, and the incubation time and temperature have been optimized to achieve the highest sensitivity. The optimized procedures for the sandwich ELISA are described in the "Methods" section. The specific reactivity of the assay was tested using meat meals prepared from different species. As shown in Figure 2, the assay clearly distinguished meat meals of bovine and ovine from other species (porcine, equine, deer, chicken, and turkey). Reciprocal dilutions of the extracts from different meat meals indicated a high sensitivity of the assay. Bovine and ovine meat meals could still be detected at levels as low as a 1:2,048 dilution of the extract. Based on an extrapolation of the dilution curves, the detection limit of the assay was below 0.025% of the extract in assay buffer. The assay consistently yielded a stronger response toward ovine (about 10 to 30% higher in the absorbance value) than bovine at all dilutions. This discrepancy between bovine and ovine was expected due to the affinity differences observed between bovine and ovine TnI. This also suggested that different calibration curves are needed for bovine and ovine if a quantitative procedure is to be employed.

Detection of bovine and ovine muscles in feed samples. A quantitative procedure was developed to determine TnI in feed samples with various levels of adulterated bovine or ovine meat meals. Calibration curves for bovine or ovine TnI were included in each of the assay plates for quantitative determination of TnI in feed samples. Profound matrix effects were observed for the three testing feeds. Therefore, individual calibration curves were established for each feed matrix to overcome these effects (Figs. 3 and 4). Among the three commercial feeds, feed A strongly

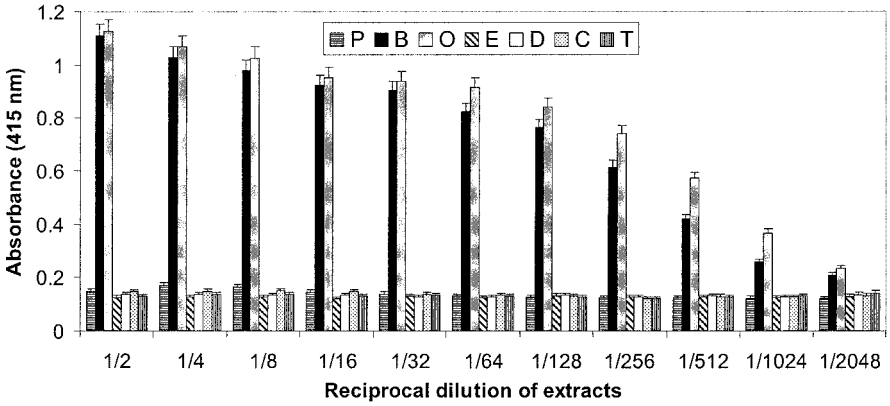

FIGURE 2. *Specific reactivity of the sandwich ELISA to meat meals produced from heated muscles (132°C/2 bar, 2 h). P, porcine; B, bovine; O, ovine; E, equine; D, deer; C, chicken; T, turkey. The assay employs MAb 5G9 as the capture antibody and biotin-labeled MAb 2G3 as the detection antibody. Values are the means of three repeated measurements, with the error bars indicating the standard deviations of each.*

diminished the assay responses, as evidenced from the slope of the calibration curves (slope = 0.0009 for bovine, 0.0012 for ovine), followed by feed C (slope = 0.0015 for bovine, 0.0021 for ovine). Feed B showed less effect on the assay response (slope = 0.0023 for bovine, 0.0028 for ovine) compared with the same concentration of TnI in the assay buffer (slope = 0.0026 for bovine, 0.0033 for ovine). Nevertheless, the feed controls without spiked TnI consistently yielded baseline readings, indicating no cross-reaction with the plant proteins in the testing feeds. The matrix effects may result from the interference of the plant proteins in the liquid phase with the binding of TnI to the capture antibody on the well surface. A linear range of the assay response was obtained for concentrations between 0 and 125 ng/ml for bovine and ovine TnIs. Samples containing TnI exceeding this range were further diluted in the corresponding feed extracts to yield a concentration within the linear range. The minimum sensitivities of the assay estimated from feed A, which displayed the highest matrix effect, were 5.0 and 4.0 ng/ml for bovine and ovine TnI, respectively.

Concentrations of TnI in the laboratory-adulterated feed samples were determined by a quantitative procedure using the respective calibration curves. The results are presented in Tables 2 and 3. TnI concentrations in the sample extracts as determined by the sandwich ELISA were proportional to the levels of adulterated bovine or ovine meat meals (0.05, 0.5, and 5% wt/wt) for all three types of feeds tested. There was no significant difference among the matrixes of the different feeds ($P > 0.05$). The coexistence of porcine, chicken, and turkey meat meals in the feeds did not affect the quantitative determination ($P > 0.05$).

DISCUSSION

This assay is the first reported monoclonal antibody-based sandwich ELISA for the simultaneous detection of rendered animal protein from bovine and ovine in feedstuffs. The sandwich ELISA reported earlier by Ansfield et al. *(5)* utilized polyclonal antibodies raised to the antigens of crude heat-stable proteins from muscle and organ tissues. False positives were observed with plant proteins in the compound animal feeds; therefore, a separate ELISA was required to identify the nonspecific reaction derived from compound animal feeds. In addition, the assays also detected proteins from dried milk powder and blood products *(6),* which are permitted ingredients in animal feeds.

The sandwich ELISA reported here utilizes MAbs raised against a defined antigen, skeletal muscle TnI, as the thermostable protein marker *(9).* In particular, the use of heat-treated TnI as the immunogen with native TnI for the hybridoma screening has ensured the selection of MAbs that recognize heat-resistant epitopes appearing in both native and heat-denatured antigen molecules. As a result, these MAbs allow the development of immunoassays based on the presence of TnI to detect muscle tissues even after high-temperature rendering processes. The use of TnI war-

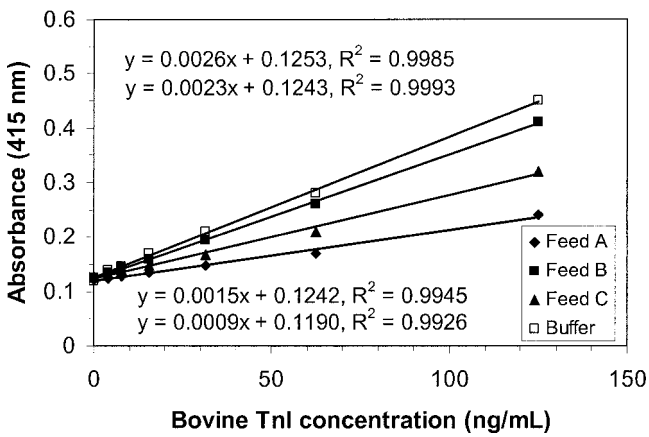

FIGURE 3. *Calibration curves of bovine TnI in assay buffer and three different feed matrixes.*

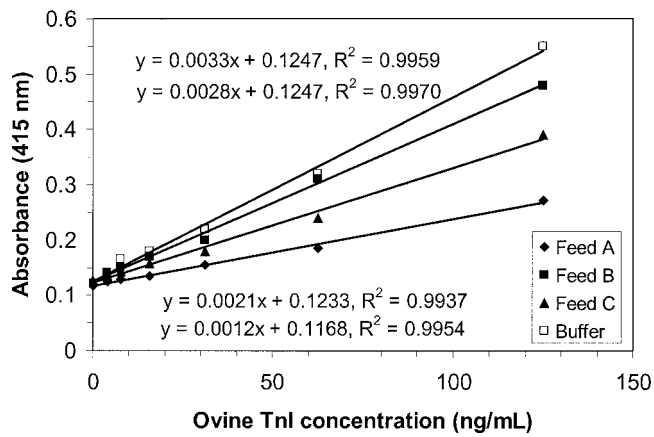

FIGURE 4. *Calibration curves of ovine TnI in assay buffer and three different feed matrixes.*

TABLE 2. *Quantitative detection of 5, 0.5, and 0.05% (wt/wt) of heat-treated (132°C/2 bar, 2 h) bovine meat meals in three different feeds with coexisting 10% porcine, chicken, or turkey meat meal*[a]

	5% bovine	0.5% bovine	0.05% bovine
10% Porcine, feed A	652 (18)	58.3 (2.3)	5.4 (3.5)
10% Porcine, feed B	638 (24)	62.8 (1.3)	5.0 (1.0)
10% Porcine, feed C	621 (21)	65.8 (4.6)	5.7 (3.2)
10% Chicken, feed A	686 (19)	64.6 (1.7)	5.3 (2.3)
10% Chicken, feed B	714 (43)	67.8 (1.4)	6.0 (0.3)
10% Chicken, feed C	635 (10)	68.6 (1.2)	7.5 (1.9)
10% Turkey, feed A	718 (33)	71.4 (2.4)	5.6 (2.9)
10% Turkey, feed B	681 (15)	71.6 (0.3)	7.6 (2.7)
10% Turkey, feed C	707 (19)	69.2 (2.2)	7.3 (2.2)

[a] Results are expressed as ng/ml of TnI in the sample extract (e.g., feed A containing 10% of porcine meal and 5% of bovine meal). Values represent the mean (standard deviation) of three repeat measurements.

TABLE 3. *Quantitative detection of 5, 0.5, and 0.05% (wt/wt) of heat-treated (132°C/2 bar, 2 h) ovine meat meals in three different feeds with coexisting 10% porcine, chicken, or turkey meat meal*[a]

	5% ovine	0.5% ovine	0.05% ovine
10% Porcine, feed A	534 (23)	57.6 (2.5)	6.3 (0.7)
10% Porcine, feed B	565 (19)	52.9 (4.0)	5.2 (0.6)
10% Porcine, feed C	592 (22)	59.5 (3.6)	7.0 (0.9)
10% Chicken, feed A	587 (43)	57.9 (3.2)	5.5 (0.8)
10% Chicken, feed B	608 (37)	56.8 (3.1)	5.1 (0.2)
10% Chicken, feed C	559 (30)	61.5 (4.6)	6.9 (0.3)
10% Turkey, feed A	598 (47)	61.0 (1.3)	7.7 (1.3)
10% Turkey, feed B	607 (24)	59.1 (3.3)	6.9 (0.8)
10% Turkey, feed C	613 (26)	61.5 (2.0)	7.5 (0.4)

[a] Results are expressed as ng/ml of TnI in the sample extract (e.g., feed A containing 10% of porcine meal and 5% of ovine meal). Values represent the mean (standard deviation) of three repeat measurements.

rants the muscle specificity of the assay. This feature, in most cases, is important in making decisions concerning the regulatory compliance of a particular feed sample in order to distinguish the prohibited animal proteins from the permitted proteins, such as those derived from milk and blood. In addition, the abundance of TnI in muscle tissue and its excellent extractability after heating offers advantages for a sensitive detection method.

This new assay requires only a simple extraction procedure, which provides greater simplicity for a qualitative assay as well as a quantitative assay if a matching matrix is provided for the calibration. In practice, many factors, such as differences in rendering conditions (time, temperature, and pressure), proportion of skeletal muscle in the meat and bone meal, and different feed matrices would likely complicate the use of calibration curves for an accurate quantification of the prohibited materials. Therefore, when an unknown sample tests positive, the bovine or ovine muscles present in the sample may be semiquantified using closely matched calibration curves for bovine and ovine TnI. Additional steps designed to remove plant proteins and to concentrate TnI may further increase the sensitivity and minimize the matrix effects of the assay.

A sandwich ELISA to detect ruminant species (bovine, ovine, and deer) simultaneously was also developed by utilizing MAb 3E12 as the capture antibody and biotin-conjugated MAb 2G3 as the detection antibody. The specific reactivity of the assay was tested using meat meals prepared from different species, as shown in Figure 5. The assay showed a clear distinction between bovine, ovine, and deer meat meals and meals from other species (porcine, equine, chicken, and turkey). Reciprocal dilutions of the extracts from different meat meals indicated that bovine, ovine, and deer could still be distinguished from other species at a dilution of 1:512, the equivalent of a detection limit of 0.2% of the extract in assay buffer. The sensitivity of this ruminant assay was a little less than that of the bovine and ovine assay due to the lower affinity of MAb 3E12. It is also possible to construct a sandwich ELISA for mammalian species (porcine, bovine, ovine, equine, and deer) by utilizing the mammalian-TnI-specific MAbs produced in our previous study *(11)*. These assays together would fulfill the analytical needs of the different regulatory requirements regarding animal proteins in a wide range of feedstuffs. The development and validation of these assays remains the highest priority for our future research.

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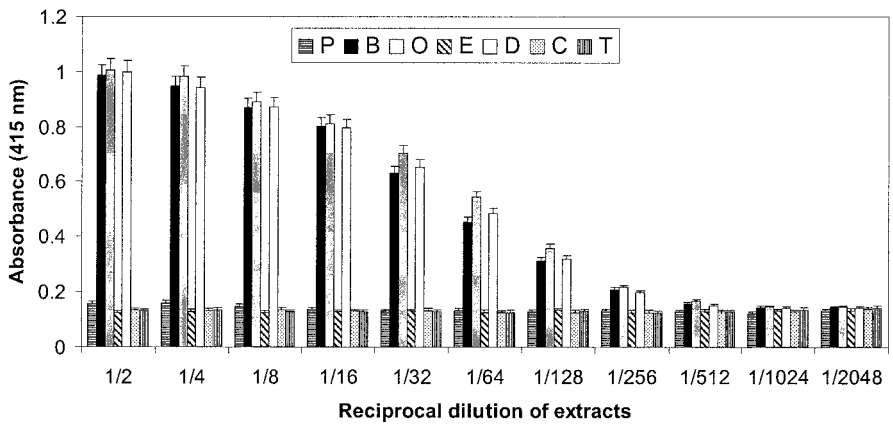

FIGURE 5. *Specific reactivity of sandwich ELISA to meat meals produced from heated muscles (132°C/2 bar, 2 h). P, porcine; B, bovine; O, ovine; E, equine; D, deer; C, chicken; T, turkey. The assay employs MAb 3E12 as the capture antibody and biotin-labeled MAb 2G3 as the detection antibody. Values are the means of three repeated measurements, with the error bars indicating the standard deviations of each.*

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