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# Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Bovine Blood in Animal Feed

JACK A. OFORI AND YUN-HWA P. HSIEH*

Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, Florida 32306-1493

The feeding of ruminant proteins to ruminants is prohibited in most countries because the practice is thought to be responsible for the spread of bovine spongiform encephalopathy. However, currently available methods to detect ruminant blood products in rendered feedstuffs are inadequate because they lack species specificity, tissue specificity, and are not based on a thermostable analyte. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed for this study that provides reliable and sensitive (0.05−0.5% v/v) detection of bovine blood materials in animal feed. The new sandwich ELISA employs two previously developed monoclonal antibodies (MAbs), Bb6G12 as the capture antibody and biotinylated MAb Bb3D6 as the detecting antibody, and is bovine-specific and blood-specific. The assay is based on the detection of a 60 kDa thermostable protein in bovine blood and provides a useful regulatory tool for monitoring fraudulent labeling or contamination of bovine blood in both heat-processed feedstuffs and unprocessed raw materials.

**KEYWORDS: Bovine; blood; monoclonal antibody; sandwich ELISA**

## INTRODUCTION

Ruminant proteins have been used as feed ingredients for ruminants to increase output for many years (*1*), but this practice has now been linked to the spread of bovine spongiform encephalopathy (BSE), commonly known as mad cow disease. As a result, many countries have enacted tough new laws banning the use of these ruminant proteins as feed ingredients for ruminants to protect both the cattle and the human population from the BSE infective agent, prions. Currently, the European Union prohibits the feeding of any protein derived from farm species back to farmed species (01/9/EC). In the United States, however, blood and blood products, milk products, and pure porcine and equine protein products are allowed to be fed to ruminants under regulation 21 CFR 589.200, although the United States is considering banning the use of blood and blood products in ruminant feed (*2*) due to recent evidence implicating blood as a carrier for prion diseases.

Because of the BSE situation, consumers are increasingly demanding beef from cattle that have been raised under natural living conditions. Thus, in addition to pasture, new kinds of plant materials are being sought to replace animal proteins in ruminant feed (*3*). There is, therefore, the need for rapid and accurate analytical methods to detect the presence of these banned or undeclared animal proteins in ruminant feed to guard against mislabeling in accordance with regulation 21 CFR 501.4 to prevent feed millers fraudulently adding these inexpensive proteins to their feed. Current methods for the detection of blood in animal feed emphasize bovine blood because blood meals used in animal feed are mostly of porcine and bovine origin, and there is so far no evidence of natural cases of prion disease in nonruminants such as pigs and horses (*4*, *5*).

To date, feed microscopy (*6*, *7*), polymerase chain reaction (PCR) (*8*−*12*), and immunochemical methods (*13*, *14*) have been developed to detect the presence of bovine blood in animal feed. These methods, however, suffer from shortcomings that make them unsuitable for the intended application. Feed microscopy is not species-specific and also requires an experienced analyst with a high degree of expertise (*7*), PCR methods tend to be affected by heat-mediated DNA degradation and are not tissue-specific (*6*), and immunochemical methods have not been demonstrated conclusively to be either tissue- or species-specific (*13*, *14*).

The objective of this study was, therefore, to develop a reliable sandwich enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies (MAbs), Bb6G12 and Bb3D6, previously obtained against bovine thermostable proteins (*15*), that is, bovine-specific to discriminate between bovine blood and blood from other species and tissue-specific to distinguish blood from nonblood tissue materials of bovine origin as well as proteins from other sources such as soy protein. The new assay is based on the recognition of a 60 kDa thermostable marker protein (analyte) that is able to withstand the heat treatment to which animal byproducts are routinely subjected.

## MATERIALS AND METHODS

**Materials.** Sodium chloride (NaCl), sodium phosphate dibasic anhydrous ($Na_2HPO_4$), sodium phosphate monobasic anhydrous ($NaH_2PO_4$), egg albumin, bovine serum albumin (BSA), sodium bicarbonate ($NaHCO_3$), sodium carbonate ($Na_2CO_3$), citric acid monohydrate, sodium dodecyl sulfate, Tween 20, 96 well polyvinyl microplate (Costar 2595), filters (Whatman #1 filter paper, syringe driven 0.22 $\mu$m and

* To whom correspondence should be addressed. Tel: 850-644-1744. Fax: 850-645-5000. E-mail: yhsieh@mailer.fsu.edu.

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0.45 μm filters), and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Gelatin was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Hydrogen peroxide ($H_2O_2$), streptavidin peroxidase polymer, NHS-CA-biotin (biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester), and ABTS (2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich Co. (St. Louis, MO). All chemicals and reagents were analytical grade. All solutions were prepared using distilled, deionized, and sterilized pure water (DD water) from the NANOpure DIamond ultrapure water system (Barnstead International, Dubuque, IA).

Bovine, sheep, horse, and rabbit blood samples were purchased from Hemostat Laboratories (Dixon, CA). Turkey, chicken, and porcine blood samples were provided by the National Renderers Association, Inc. (Alexandria, VA). Spray-dried bovine and porcine plasma meals were obtained from Merrick's Inc. (Middleton, WI), and whole bovine blood powder was from California Spray Dry Co. (Stockton, CA). Meat bone meals (of porcine, bovine, and ovine origins), feather meal, and dairy blend were obtained from companies who prefer not to have their identity disclosed here. Soy protein concentrate was purchased from Central Soya Co., and nonfat dry milk was from a local supermarket in Tallahassee, FL. Beef loin, lamb shoulder, pork loin, frozen dressed rabbit, turkey breast, and whole chicken were purchased from local supermarkets in Tallahassee, FL. Horse meat was obtained from the veterinary school at Auburn University (AL).

**MAbs.** The MAbs used in this study, Bb6G12 and Bb3D6, both belong to the IgG1 subclass and were previously developed against thermostable bovine blood proteins in our laboratory. These two antibodies were selected for the new sandwich ELISA because they recognize the same antigenic protein (~60 kDa) in both raw and heat-treated bovine blood, as revealed by Western blot analysis, and also bind complimentarily to this common antigenic protein as determined by the method described by Friguet and others (*16*) as described previously (*15*). Both MAbs Bb6G12 and Bb3D6 were purified from mouse ascites fluid using a Protein A affinity column on an Econo low-pressure chromatography system (Bio-Rad Laboratories Inc., Hercules, CA) in accordance with the manufacturer's instructions. MAb 3D6 was conjugated with biotin using NHS-CA-biotin following a standard procedure (*17*). Concentrations of purified MAb Bb6G12 IgG and biotin conjugated MAb Bb3D6 were determined by UV spectrophotometer (SmartSpec 3000, Bio-Rad) at 280 nm.

**Preparation of Blood Protein Extracts.** Blood samples were stored at −80 °C prior to sample preparation. Twenty milliliters of blood in a 50 mL beaker was covered with aluminum foil and heated in a boiling water bath for 15 min or autoclaved for 15 min at 121 °C to obtain the "cooked" and "autoclaved" blood samples, respectively. Each heat-treated blood sample was then mashed into fine particles using a glass rod. An equal volume (20 mL) of the extraction buffer (10 mM phosphate-buffered saline, PBS) was added to the mashed samples to extract the soluble TSPs, and the mixture was homogenized for 2 min at 11000 rpm using the ULTRA-TURRAX T25 basic homogenizer (IKA Works Inc., Wilmington, NC). The homogenized samples were allowed to stand for 2 h at room temperature followed by another 2 h at 4 °C. The mixtures were then centrifuged (Eppendorf 5810R centrifuge, Brinkman Instruments Inc., Westbury, NY) at 3220*g* for 60 min at 4 °C, and the supernatants were filtered through Whatman #1 filter paper and stored at −20 °C until use. Raw blood samples were used without further treatment.

**Preparation of Other Protein Extracts.** *Flesh Protein Extracts.* Fat and connective tissues were trimmed off meat samples (beef, pork, lamb, rabbit, horse, chicken, and turkey). The lean meat samples were then cut up and ground twice using a meat grinder (Waring Consumer Products, East Windsor, NJ) to ensure thoroughness and homogeneity. Ten gram (10 g) minced meat samples from each species were weighed into beakers. The beakers were covered with aluminum foil and autoclaved for 15 min at 121 °C. Another portion (10 g) of minced meat sample from each species was prepared in a similar fashion to the autoclaved meat but heated in a boiling water bath for 15 min. The samples were then prepared in the same manner as the blood samples described above. The raw beef extract was prepared by adding 10 mL of 10 mM PBS to 10 g of minced raw beef in a sampling bag and blending the mixture with a stomacher (model #STO 400, Tekmar Co., Cincinnati, OH) for 60 s followed by standing at room temperature for 2 h plus another 2 h at 4 °C. The mixture was then centrifuged, filtered as described above, and stored at −20 °C.

*Nonflesh Protein Extracts.* The nonflesh proteins included gelatin, soy protein concentrate, egg albumin, nonfat dry milk, and BSA. For each, 2 g of the sample was placed in a beaker and 10 mL of extraction buffer (10 mM PBS) was added. The mixtures were then held, centrifuged, and filtered as described above. All of the clear protein extracts were stored at −20 °C until used for the sandwich ELISA.

*Commercial Feedstuff Extracts.* To 2 g each of spray-dried bovine plasma, whole bovine blood powder, spray-dried porcine plasma, meat bone meals of bovine, porcine, and ovine origins, feather meal, and dairy blend in a beaker was added 10 mL of extraction buffer (10 mM PBS). The mixtures were extracted, centrifuged, filtered, and stored as described previously.

**Preparation of Laboratory-Adulterated Samples.** Adulterated samples were prepared by mixing bovine blood with porcine blood at nine levels, 0, 0.01, 0.05, 0.1, 0.5, 1, 3, 5, and 10% (v/v), using a minishaker (MS 1 Minishaker, IKA Works, Inc., Wilmington, NC). The mixture was autoclaved at 121 °C for 15 min. Another set of samples was similarly mixed and heated in a boiling water bath for 15 min. The heat-treated samples were finely mashed, and an equal part of extraction buffer (10 mM PBS) was added to extract the soluble proteins. The mixtures were then held, centrifuged, filtered, and stored as previously described. Porcine blood containing no bovine material (0%) was included as a negative control.

Spray-dried bovine plasma was separately mixed with spray-dried porcine plasma, and also whole bovine blood powder mixed with spray-dried porcine plasma at 0, 0.1, 0.5, 1, 5, and 10% adulteration levels. Five-fold (v/w) of the extraction buffer (10 mM PBS) was then added to each mix. The mixtures were held, centrifuged, filtered, and stored as previously described. Spray-dried porcine plasma or whole porcine blood powder containing no bovine material (0%) was included as a negative control.

**Sandwich ELISA.** Optimization was performed initially to ascertain which antibody to use for capture and which for detection, the optimum dilution for each antibody, the optimum incubation period, and the best choice of blocking buffer. On the basis of the optimization results (data not shown), MAb Bb6G12 was selected for coating on the microplate as the capture antibody and biotin-conjugated MAb Bb3D6 as the detection antibody. One hundred microliters (100μL) of the capture antibody (Bb6G12 purified IgG) diluted 1:1000 in PBS, corresponding to 0.18 μg protein per 100 μL per well, was coated on the wells of the microplate and incubated at 37 °C for 2 h. The plate was washed three times with PBST and incubated for 1 h at 37 °C with 200 μL of blocking buffer (1% BSA−PBS). After the plate was washed twice with PBST, 100 μL of the controls and protein samples appropriately diluted in antibody buffer was added to the plate and the plate was incubated for 2 h at 37 °C. The plate was washed three times and incubated for 2 h at 37 °C with 100 μL of the detection antibody (biotin-conjugated Bb3D6) diluted 1:1000 in antibody buffer, corresponding to 0.175 μg protein per 100 μL. The plate was again washed three times and incubated for 1 h at 37 °C with 100 μL of the enzyme, streptavidin peroxidase polymer diluted 1:3000 in antibody buffer. After another washing step, the plate was incubated with 100 μL of enzyme substrate (22 mg of ABTS and 15 μL of 30% $H_2O_2$ in 100 mL of 0.1 M phosphate citrate buffer, pH 4.0) for 20 min at 37 °C to develop the color. The enzyme reaction was stopped by the addition of 100 μL of 0.2 M citric acid, and the absorbance was read at 415 nm using a microplate reader (Bio-Rad, model 450).

**Statistical Analysis.** All experiments were performed in triplicate and repeated twice. Data were analyzed using Microsoft Excel 2000. A paired *T*-test was used to determine the detection limit by comparing the differences in the ELISA readings between the background (0%) and laboratory-adulterated samples. $P \leq 0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

**Specificity of the Sandwich ELISA.** The species specificity of the sandwich ELISA using MAb Bb6G12 as the capture

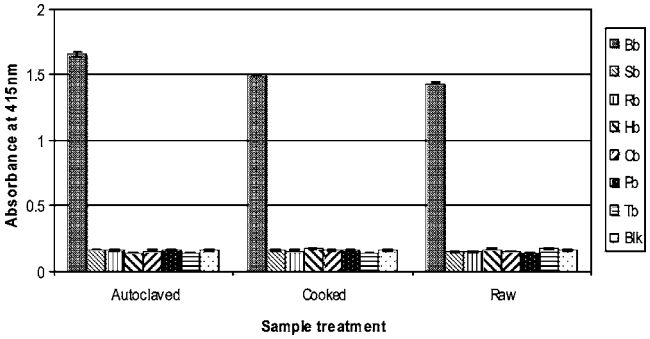


**Figure 1.** Species specificity of sandwich ELISA using purified MAb Bb6G12 as the capture antibody and biotin-conjugated MAb Bb3D6 as the detection antibody. Soluble proteins extracted from heat-treated blood samples and raw blood samples were diluted 1:3 in 1% BSA–PBST. Bb, bovine blood; Sb, sheep blood; Rb, rabbit blood; Hb, horse blood; Cb, chicken blood; Pb, porcine blood; and Tb, turkey blood. Results are expressed as $A_{415} \pm$ SD; $n = 3$.

antibody and MAb Bb3D6 as the detection antibody is shown in **Figure 1**. In this sandwich assay, protein extracts from cooked and autoclaved blood samples and raw whole blood diluted 1:3 in antibody buffer were added to microplates precoated with MAb Bb6G12. The assay reacted strongly with raw, autoclaved, and cooked bovine blood but did not react with raw, cooked, or autoclaved blood from any of the other species tested. The readings observed for both heat-treated (cooked and autoclaved) and raw bovine blood were about the same, indicating that this assay works equally well with raw bovine blood as with heat-treated samples. The readings for nonbovine blood samples were about the same or lower than the blank. Both combinations, MAb Bb6G12 as capture antibody and MAb Bb3D6 as detection antibody and vice versa, were tested during the assay development, and the results were the same.

The developed sandwich assay is the first MAb-based immunoassay that has desired species specificity and reactivity in both raw and heat-treated blood samples. Polo and others (*14*) developed an immunodiffusion method for detection of bovine blood contamination in porcine blood in both liquid (raw blood) and spray-dried (mild heat-treated blood) blood. The sensitivity was higher in liquid samples as compared to spray-dried samples, which indicates that the assay performance was hampered by mild heat treatment of the sample. The authors suggested that their method would be best applied to screening tests in slaughterhouses, where liquid samples are more common. Newgard and others (*13*), on the other hand, developed a lateral flow immunoassay device using commercially produced goat anti-bovine IgG antibodies, which need to be affinity absorbed with porcine IgG to remove the cross-reactivity with porcine. They tested only spray-dried samples and hence did not determine whether or not their assay would also react with raw bovine blood or blood samples from other species.

**Cross-Reactivity with Other Proteins.** Cross-reactivity of the assay with other proteins, namely, the flesh proteins (muscle) from bovine and nonbovine species, and nonflesh proteins (soy, egg albumin, etc.) that are commonly added to animal feed, are presented in **Figure 2a**,**b**. The assay did not react with any of the other nonblood proteins tested except for a weak cross-reaction with nonfat dry milk. This weak cross-reaction with either MAb was not shown when an indirect ELISA format was used (*15*); this difference may be due to the much higher antigen

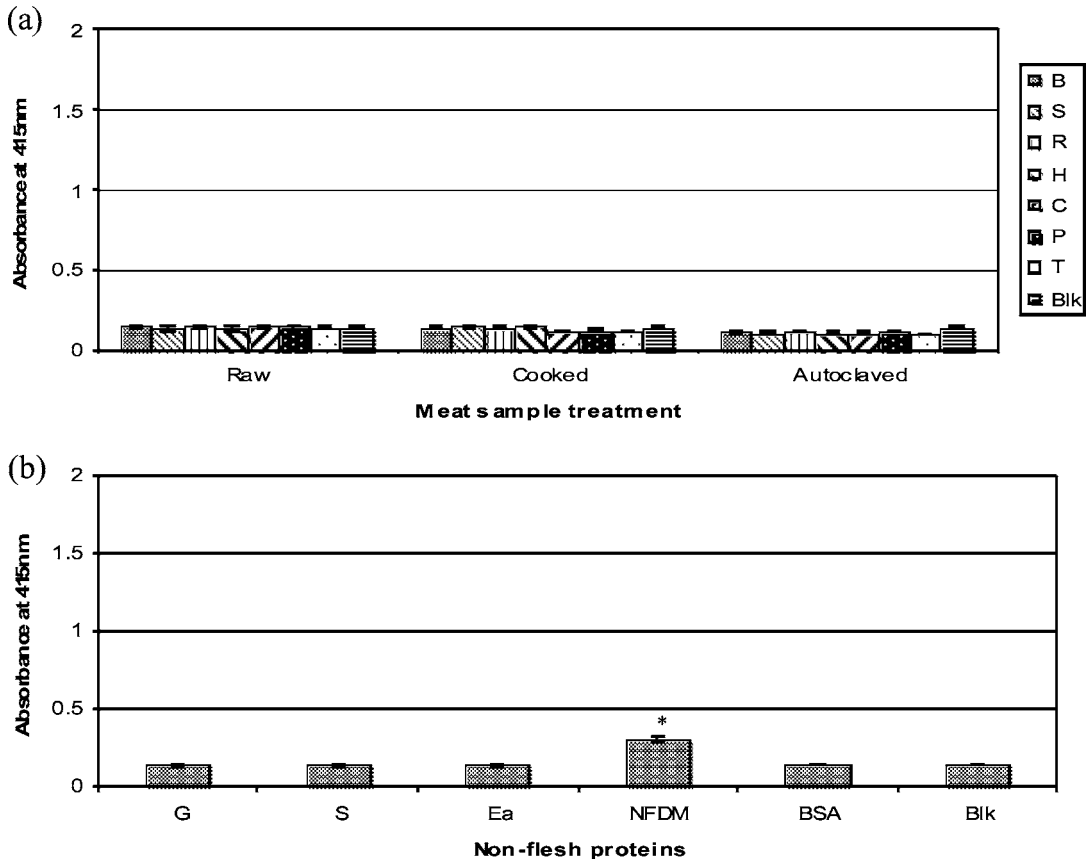


**Figure 2.** Cross-reactivity of sandwich ELISA with (**a**) flesh (meat) proteins and (**b**) nonflesh proteins using purified MAb Bb6G12 as the capture antibody and biotin-conjugated MAb 3D6 as the detection antibody. Soluble proteins extracted from meat samples were diluted 1:3 in 1% BSA–PBST. B, beef; S, sheep; R, rabbit; H, horse; C, chicken; P, pork; T, turkey; G, gelatin; S, soy protein concentrate; Ea, egg albumin; and NFDM, nonfat dry milk. Results are expressed as $A_{415} \pm$ SD; $n = 3$. An asterisk indicates significant difference ($P < 0.05$).

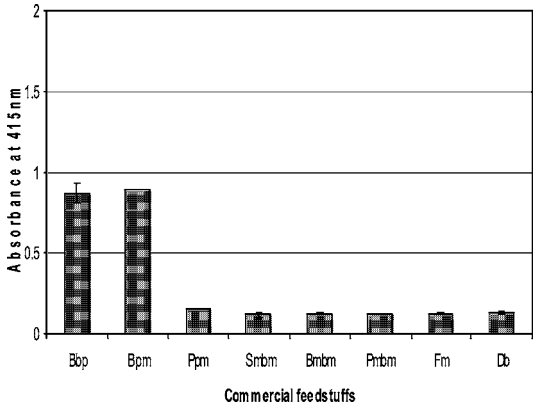


**Figure 3.** Performance of sandwich ELISA with commercial feedstuffs using purified MAb Bb6G12 as the capture antibody and biotin-conjugated MAb Bb3D6 as the detection antibody. Soluble proteins extracted from the feedstuff samples with 10 mM PBS were diluted 1:3 in 1% BSA–PBST. Bbp, whole bovine blood powder; Bpm, spray-dried bovine plasma; Ppm, spray-dried porcine plasma; Smbm, ovine meat bone meal; Bmbm, bovine meat bone meal; Pmbm, porcine meat bone meal; and Fm, feather meal. Results are expressed as $A_{415} \pm$ SD; $n = 3$.

concentration in the sample extracts added to the antibody precoated microplates for the sandwich ELISA than that used for indirect ELISA, where only 0.5 $\mu$g protein per well of protein extracts was coated on the plate. Also, some proteins are common to both blood and milk and have similar molecular structures, such as albumins of bovine blood and serum, which are identical, and transferrins, although these latter are found in larger quantities in sera than in milk. Because the nonfat dry milk was of bovine origin, it is likely that the antibodies react with milk proteins that have a similar structure but are present at a very low concentration in milk. This trace reactivity against nonfat dry milk, however, can be easily eliminated when the dilution of the antibodies is increased (data not shown).

**Performance with Commercial Feedstuffs.** The performance of the sandwich ELISA with commercially produced feedstuffs is shown in **Figure 3**. The assay reacted only with spray-dried whole bovine blood and spray-dried bovine plasma. The assay did not react with the other feedstuffs tested, including feather meal and MBMs. This result further demonstrates the tissue specificity of the sandwich ELISA, namely, its ability to discriminate between bovine blood and bovine muscle. In addition, the assay neither reacted with the bovine MBM nor with the bovine meat extracts, suggesting that the assay reacts with added bovine blood but not with residual blood because both the bovine MBM and the bovine meat contained residual blood. This is probably because the residual blood is present at levels that are below the detection limit of the assay.

**Detection Limit of the Assay.** The limit of detection was defined as the smallest quantity of the analyte that could be significantly ($P \leq 0.05$) distinguished from background (0% adulteration level) in the assay. The detection limit for the assay was 0.05% (v/v) for both autoclaved bovine blood in porcine blood (**Figure 4a**) and cooked bovine blood in porcine blood (**Figure 4b**) samples. The detection limit was 0.1% (w/w) in spray-dried bovine plasma in spray-dried porcine plasma (**Figure 5a**). For whole bovine blood powder in spray-dried porcine plasma (**Figure 5b**), the detection limit was 0.5% (w/w). The observed difference in detection limit for whole bovine blood powder as compared to the spray-dried bovine plasma may arise as a result of differences in the manufacturing procedures. The manufacturing of spray-dried bovine plasma involves an additional centrifugation step that precipitates out blood cells and

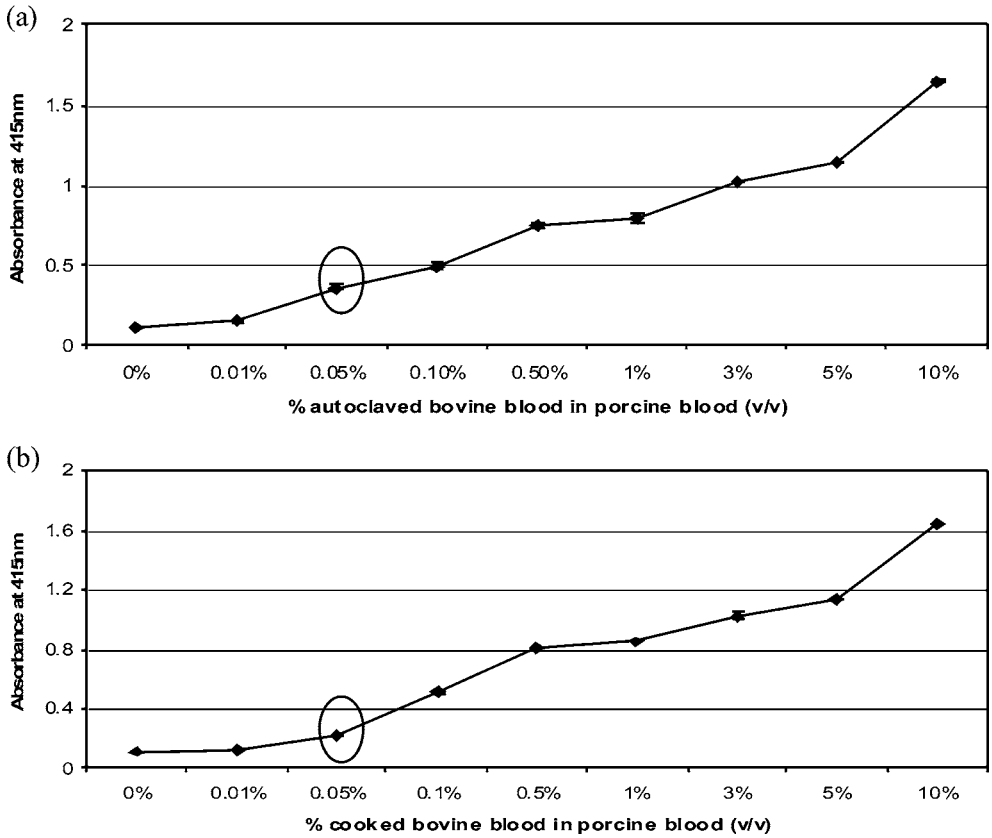


**Figure 4.** Detection limit of (**a**) autoclaved bovine blood in autoclaved porcine blood and (**b**) cooked bovine blood in cooked porcine blood using sandwich ELISA with purified MAb Bb6G12 as the capture antibody and biotin-conjugated MAb Bb3D6 as the detection antibody. Soluble proteins were extracted from autoclaved and cooked bovine blood in porcine blood mixtures. Results are expressed as $A_{415} \pm$ SD; $n = 3$.

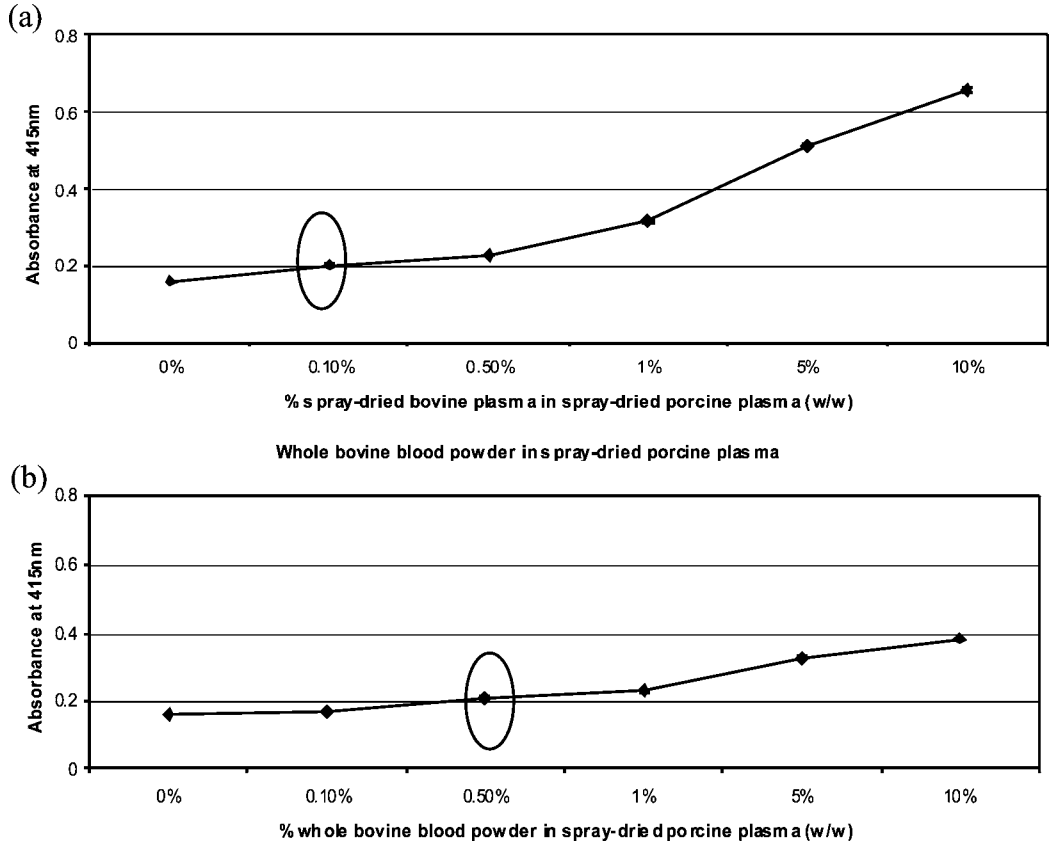


**Figure 5.** Detection limit of (**a**) spray-dried bovine plasma in spray-dried porcine plasma and (**b**) whole bovine blood powder in spray-dried porcine plasma using sandwich ELISA with purified MAb Bb6G12 as the capture antibody and biotin-conjugated MAb Bb3D6 as the detection antibody. Soluble proteins extracted from spray-dried porcine plasma mixed appropriately with spray-dried bovine plasma. Results are expressed as $A_{415} \pm$ SD; $n = 3$.

some of the proteins in the blood. Thus, for a given amount of spray-dried bovine plasma or whole bovine blood powder, the concentration of the target soluble antigenic molecule will be higher in the former as compared to the latter. The amount of antibody that is bound is increased (and hence the signal) when the amount of antigen is increased (*17*). The higher concentration of the antigen in the spray-dried bovine plasma as compared to the whole bovine blood powder may have led to an increase in antibody binding, ultimately lowering the detection limit for spray-dried bovine plasma as compared to whole bovine blood powder. The detection limit for autoclaved and cooked bovine blood (0.1% v/v) (**Figure 4a**,**b**), both of which underwent centrifugation during preparation, was the same as that for spray-dried bovine plasma, reinforcing the assertion that differences in sample types were responsible for the difference in detection limit observed for spray-dried bovine plasma and whole bovine blood powder. Similar results were obtained by Polo and others (*14*), who reported a detection limit of 0.3% (v/v) bovine plasma in porcine meal and 0.5% (v/v) whole bovine blood in porcine plasma. The detection limit of our sandwich ELISA for spray-dried bovine plasma in porcine plasma (0.1% v/v) is lower than the detection limit (0.3% v/v) of the assay using anti-IgG polyclonal antibodies reported by Polo and others (*14*).

**Validation of the Assay.** The assay was also validated in terms of its reproducibility, sensitivity, specificity, and overall accuracy (**Table 1**). Reproducibility is defined as the variability between replicate determinations in the same assay (intra-assay variability) and in different assays (interassay variability) and is generally represented by the coefficient of variation (CV) (*18*). This was computed using bovine blood-containing samples (spray-dried bovine plasma, whole bovine blood powder, autoclaved, cooked, and raw bovine blood) at levels above the detection limit of the assay. The assay was found to have an intra-assay variability of 0.19−1.15%, an interassay variability of 0.88−2.31%, and an overall variability of 1.28%. Sensitivity is defined as the ability of the test to detect positive samples as positive and is computed as $A/B \times 100\%$, where $B$ is the number of positive samples tested and $A$ is the number of positive samples that the test is able to correctly identify as positive (*18*). Out of the 70 positive samples tested, the assay correctly identified all 70 as positive. The assay thus had a sensitivity of 100%. The specificity is defined as the ability of the test to detect negative samples as negative and is computed as $C/D \times 100\%$, where $D$ is the number of negative samples tested and $C$ is the number of negative samples that the test is able to correctly identify as negative (*18*). This was done using nonbovine-containing samples (autoclaved and cooked blood from other species, spray-dried porcine plasma, meat bone meals, and feather meal). Out of 108 negative samples tested, the assay correctly identified all 108 as negative. The assay thus had a specificity of 100%. The assay had an overall accuracy, which was calculated as the combined ability of the test to correctly detect positive and negative samples (overall accuracy = specificity and sensitivity), of 100% (**Table 1**).

**Table 1.** Validation of Assay

| | |
|---|---|
| interassay variability (%) | 0.19–1.15 |
| intraassay variability (%) | 0.88–2.31 |
| overall variability (%) | 1.28 |
| sensitivity (%) | 100 |
| specificity (%) | 100 |
| overall accuracy (%) | 100 |

In conclusion, this MAb-based immunoassay is bovine-specific, discriminating between bovine blood and blood from

all of the other species considered. The assay is based on the detection of a thermostable biomarker protein that can be recognized in raw, cooked, and autoclaved bovine blood and is also tissue-specific, distinguishing between blood and other tissues such as muscle and gelatin of bovine origin. In addition, this sandwich ELISA did not react with the food proteins, such as soy and egg albumin, that are commonly added to feedstuffs except for a trace nonspecific reactivity against nonfat dry milk if the concentration is high. The assay had good performance characteristics, as evidenced by a detection limit of 0.1% (v/v) spray-dried bovine plasma in spray-dried porcine plasma, a detection limit of 0.5% (v/v) whole bovine blood powder in spray-dried porcine plasma, an overall accuracy of 100%, and an overall variability of 1.28%. This study reports the first MAb-based assay for the sensitive and reliable detection of bovine blood in a wide variety of processed and raw feed materials.

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