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Monoclonal Antibodies Specific to Thermostable Proteins in Animal Blood

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Bovine plasma proteins are used as high-quality protein supplements in animal feed and as binders or colorants in food for human consumption. Religious observance, as well as recent fears of epidemic bovine spongiform encephalopathy, highlights the need for methods to detect bovine blood in processed food and animal feed for regulatory purposes, as the currently available methods are neither species-specific, blood-specific, nor valid for excessively heat-processed samples. This paper reports the development of monoclonal antibodies (MAbs) raised against bovine thermostable plasma proteins that display a unique species specificity pattern for plasma proteins. Immunoblotting revealed several thermostable antigenic proteins (10, 25, 40, and 60 kDa) in bovine plasma sterilized at 121 °C for 15 min. These MAbs can be employed individually or combined in immunoassays for analytical purposes and investigations of the chemical and biological properties of the thermostable plasma proteins identified here.

KEYWORDS: Bovine blood; thermostable plasma protein; monoclonal antibody; immunoassay

INTRODUCTION

Blood detection has been important for forensic science and clinical pathology investigations for decades, and several presumptive tests have been developed to identify bloodstains on the basis of color changes because of the oxidation of specific chemical reagents by the blood pigment, hemoglobin, in the presence of peroxide; a positive reaction is presumed to be blood (*1*). However, these tests are not blood specific as many chemical oxidants such as perchlorates and permanganates, chromates and dichromates, and copper and iron salts also cause oxidation of these reagents, producing characteristic color changes even when no hemoglobin is present (*2*). Improved methods for blood detection are now available, including spectrophotometry (*2*−*4*), the Takayama confirmatory test (*5*), and immunochemical methods (*6*−*8*) that are mainly based on heat-labile blood proteins such as hemoglobin and immunoglobulins as the analyte. These assays are not species-specific and hence do not discriminate between animal species and are also not suitable for the detection of blood in heat-treated samples such as cooked food or the blood meal in animal feed.

In many countries, animal blood is used as a source of human food, usually in the form of blood sausages, pudding, soup, bread, or crackers (*9*). Although not popular in the United States, blood finds its way into the human food chain in various forms. Because of the superior functionality of its plasma proteins, blood is used in sausage products to enhance color (*9*, *10*), in bakery products as an egg substitute, or in liquid foods as a clarifying agent (*9*, *11*). Spray-dried whole blood and plasma proteins have commonly been utilized as high-quality protein ingredients in feed for both pets and farm animals (*8*). In addition, blood protein is also found in laboratory, medical, industrial, and fertilizer formulations (*9*, *12*).

With the advent of the fatal epidemic prion disease bovine spongiform encephalopathy (BSE), animal proteins including blood have been banned in animal feed in European countries to prevent its spread. The new variant Creutzfeldt−Jakob disease (nvCJD) in humans has been linked to the consumption of beef infected with BSE. Since the infectious prion proteins are extremely resistant to heat, ultraviolet light, and ionizing radiation, normal sterilization processes and common disinfectants do not destroy prions (*13*−*15*). Recent evidence indicates that blood also carries some level of infectivity for transmissible spongiform encephalopathies (TSEs). Transmission of TSE through inoculation of blood has been demonstrated experimentally in animals infected with various strains of TSEs (*16*−*18*). Consequently, both humans and animals are potentially exposed to the infectious agent as a result of the various uses of bovine blood because the processing involved in the manufacture of these blood-containing materials fails to inactivate the infectious agent as a result of their hardy nature. In addition, some sensitized individuals are allergic to blood proteins, while others avoid blood consumption for religious or ethical reasons.

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There is therefore an urgent need for suitable methods to detect the presence of blood, especially ruminant blood as BSE has never been found in nonruminant animals, in both raw and heat-processed materials for labeling and regulatory enforcement. However, analytical methods that are species-specific and blood-specific and that are based on the detection of a thermostable plasma protein as the analyte are not currently available. Since antibody-based immunoassays are well-recognized and are widely used in food and agricultural areas as a convenient and economical analytical or research tool, we attempted to develop an improved immunoassay using MAbs for blood detection. In this study, we developed a panel of species-specific monoclonal antibodies capable of recognizing thermostable proteins in animal blood. These MAbs can be employed individually or in combination in various formats of immunoassays for a wide range of applications.

MATERIALS AND METHODS

Materials. Tris-buffered saline, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl (pH 8.8), TEMED (*N,N,N,N′*-tetra-methyl-ethylenediamine), Precision Plus Protein Kaleidoscope Standards, 30% acrylamide/bis solution, Tris/glycine buffer, 10 × Tris/glycine/SDS buffer, supported nitrocellulose membrane (0.2 μm), and thick blot paper were obtained from Bio-Rad Laboratories Inc., Hercules, CA. Hydrogen peroxide, aminopterin-hypoxanthine-thymidine (HAT) medium, streptavidin peroxidase polymer, horseradish peroxidase conjugated goat antimouse IgG (Fc specific), NHS-CA-Biotin (biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester), ABTS (2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid), and β-mercaptoethanol were purchased from Sigma-Aldrich Co., St. Louis, MO. Brom phenol blue sodium salt was purchased from Allied Chemical Corporation, New York. All other chemicals and reagents, molecularporous membrane tubing (MWCO: 6−8000), and filters (Whatman No. 1 paper, syringe driven 0.22 μm, and 0.45 μm filters) were purchased from Fisher Scientific, Fair Lawn, NJ. All solutions were prepared using distilled deionized pure water (DD water) from a NANOpure DIamond ultrapure water system (Barnstead International, Dubuque, IA).

Bovine, sheep, horse, and rabbit blood were purchased from Hemostat Laboratories, Dixon, CA. Fresh turkey, chicken, and porcine blood was provided by the National Renderers Association. Fresh 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 School of Veterinary Medicine, Auburn University. Spray-dried bovine and porcine plasma meals were obtained from Merrick's Inc., Middleton, WI, and whole bovine blood powder was obtained from California Spray Dry Company, Stockton, CA. Soy protein concentrate was purchased from Central Soya Company, Fort Wayne, IN, and nonfat dry milk (NFDM) was purchased from a local supermarket in Tallahassee, FL.

Sample Preparation. *Extraction of Thermostable Soluble Proteins (TSPs) from Animal Blood.* Soluble TSPs were extracted from cooked and autoclaved whole blood from each animal species. Twenty milliliters of whole blood in a 50 mL beaker was covered with aluminum foil and was heated in a boiling water bath for 15 min or was 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 11 000 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 No. 1 filter paper. The protein concentrations of the clear filtrates were determined using protein assay kit II (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the standard in this assay. All protein extracts were stored at −20 °C until use.

Protein Extractions from Nonblood Samples and Commercial Feedsuffs. 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 were ground twice using a meat grinder (Waring Consumer Products, East Windsor, NJ) to ensure thoroughness and homogeneity. Ten grams (10 g) of minced meat samples from each species was weighed into beakers. The beakers were covered with aluminum foil, were sealed with adhesive tape, and were autoclaved for 15 min at 121 °C. Another portion (10 g) of minced meat sample from each species was prepared in a similar fashion as the autoclaved meat but was heated in a boiling water bath for 15 min. The autoclaved and cooked samples were then mashed into fine particles using a glass rod. Ten milliliters (10 mL) of extraction buffer (10 mM PBS) was then added to the mashed samples, and the mixture was then homogenized for 2 min at 11 000 rpm. The homogenized sample was allowed to stand at room temperature for 2 h followed by another 2 h at 4 °C. The mixtures were then centrifuged at 3220*g* for 60 min at 4 °C. The cooked and autoclaved flesh protein extract supernatants were filtered through Whatman No. 1 filter paper and were stored at −20 °C until used. 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 by blending the mixture with a stomacher (Model Number STO 400, Tekmar Company, 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 and filtered as described above and was stored at −20 °C. The nonflesh proteins included gelatin, soy protein concentrate, egg albumin, NFDM, and BSA. To 2 g of each of the nonflesh proteins, the commercial spray-dried bovine plasma, and the whole bovine blood powder in a beaker was added 10 mL of 10 mM PBS to extract the soluble proteins. The mixtures were then held, centrifuged, and filtered as described above. All the clear protein extracts were stored at −20 °C until being used for the indirect enzyme-linked immunosorbent assay (ELISA).

Production of MAbs. *Immunization.* The autoclaved bovine blood TSP crude extract was dialyzed in 10 mM PBS for 24 h with frequent changes, and the dialyzed extract was used as the immunogen. Three female BALB/c mice were immunized subcutaneously with 100 μg/mouse of the immunogen emulsified with an equal volume of Freund's complete adjuvant. Three booster injections prepared in the same manner using Freund's incomplete adjuvant were administered to each mouse at 4-week intervals. Test sera from the mice were collected 8 days after each boosting by tail bleeding. The titer of the sera was determined by indirect ELISA. The mouse showing the highest titer was injected intraperitoneally with 100 μg of the immunogen in PBS 4 days prior to fusion. The immunization and the following hybridoma procedures were performed in the Hybridoma Facility at Auburn University, Auburn, AL, in compliance with the University's Animal Welfare guidelines.

Hybridoma Procedures. The spleen cells from the immunized mice were fused with murine myeloma cells (P3 × 63.Ag8.653, ATCC CRL 1580) at a ratio of 5:1 in the presence of polyethylene glycol (MW 4000) for hybridoma production. The general procedures described by Köhler and Milsten (*19*) were followed with modifications and the following specific screening procedures. The cells were diluted to an appropriate density and were cultured in aminopterin-hypoxanthine-thymidine (HAT) medium. The medium was changed twice to remove residual antibodies before the initial screening against the immunogen (autoclaved bovine blood TSPs) using indirect ELISA. Those positive hybridomas were selected, were cloned twice by limiting dilution, and were expanded. For a secondary selection, the expanded positive hybridomas were screened for reactivity with the native blood serum proteins, TSPs from the cooked blood samples from bovine and other animal species, and various tissue protein extracts. Because IgM antibodies are generally more difficult to purify and store, only the IgG class of MAbs were selected. This was achieved by using an IgG γ-chain specific secondary antibody as a probe in the ELISA screening procedures. MAbs were obtained in supernatants from the propagated cell cultures. The isotype of each selected MAb was determined with

a mouse MAb isotyping kit (ISO-2 1 kit, Sigma) according to the manufacturer's protocol.

Characterization of MAbs. *Indirect ELISA.* One hundred microliters (100 μL) of each properly diluted sample protein extract and control containing 0.5 μg of soluble protein in 0.06 M carbonate buffer (pH 9.6) was coated into the wells of a 96-well polyvinyl microplate (Costar 2595, Fisher) and was incubated at 37 °C for 2 h. The plate was then washed three times with PBST [0.05% v/v Tween-20 in 10 mM PBS, pH 7.2] and was incubated with 200 μL/well blocking solution (3% NFDM in PBS) at 37 °C for 2 h, followed by another washing step. The undiluted or appropriately diluted MAb supernatants (100 μL) in antibody buffer [1% w/v BSA in PBST] were added to each well and were incubated at 37 °C for 2 h. After washing three times with PBST, diluted (1:3000 in antibody buffer) horseradish peroxidase-conjugated goat antimouse IgG-Fc specific solution was added. The plate was incubated at 37 °C for 2 h and then was washed five times before the addition of the substrate solution (22 mg of ABTS and 15 μL of 30% hydrogen peroxide in 100 mL of 0. 1 M phosphate-citrate buffer, pH 4.0). The color was developed at 37 °C for 20 min. The enzyme reaction was stopped by adding 0.2 M citric acid solution, and the absorbance was measured at 415 nm using a microplate reader (Model 450, Bio-Rad). This technique was used to test titers of the antisera, to screen hybridoma clones, and to test each MAb for immunoreactivity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot. SDS-PAGE was performed to resolve the soluble TSPs of the different blood sample extracts according to the method of Laemmli (*20*) with modifications. Briefly, soluble proteins (3 μg of protein in 15 μL per lane) from the samples were loaded on 5% stacking gel (pH 6.8) and were separated on a 12% polyacrylamide separating gel (pH 8.8). The gel was subjected to electrophoresis at 200 V for 45 min using a Mini-Protein 3 electrophoresis cell (Bio-Rad, 161-3301) connected to a power supply (Model 3000, Bio-Rad). A Western blot test was then carried out according to the method by Towbin et al. (*21*) with modifications to determine the molecular weights of the immunogenic components that reacted with each MAb. After separation of the proteins on the polyacrylamide gel by means of SDS-PAGE, protein bands were transferred electrophoretically (1 h at 100 V) from the gel to nitrocellulose membranes using a MiniTrans-Blot unit (Bio-Rad). Upon completion of the transfer, the membrane was washed with TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5), was blocked with 1% BSA in TBS, and then was incubated with the undiluted MAb supernatant overnight at 4 °C. The excess antibody was removed by washing with TBST, and the membrane was incubated with goat antimouse IgG-alkaline phosphatase conjugate diluted 1:3000 in antibody buffer for 1 h at room temperature. After washing, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitroblue tetrazolium chloride (BCIP/NBT) in 0.1 M Tris buffer at pH 9.5 to develop the color. The color reaction was stopped by washing the membrane with distilled water. The appearance of a dark purplish band indicated an antibody binding site. Prestained broad range protein standards (Precision Plus Protein Kaleidoscope Standards, Bio-Rad, 161-0375) were used as molecular weight markers for both the SDS-PAGE and Western blot.

RESULTS AND DISCUSSION

Production of the MAbs. From two consecutive fusions, the overall positive rate was approximately 10% of the original fusion wells when first screened against the crude bovine blood immunogen. After the supernatant from the positive wells was retested against heterologous blood protein extracts, eight cell lines secreting MAbs with distinct patterns were selected on the basis of their reactivity and unique reaction pattern.

In this study, crude protein extract from autoclaved bovine blood was used as the immunogen to develop the MAbs. Autoclaved blood was used to ensure that the soluble molecules obtained in the extract were stable even after severe heat treatment. One major advantage of the hybridoma technology is that the crude antigen can be used as the immunogen and that the desired specificity of the MAbs can be selected during the hybridoma screening stages. Although in most cases, especially when the target antigen is defined and available, using pure antigen increases the chances of developing the desired MAbs, for this study there was no prior knowledge of potentially suitable thermostable, species-specific blood antigens, so immunizing a crude TSP increased the chance of producing a diverse set of MAbs targeted to different thermostable antigens. Any of the individual TSPs in the mixture were consequently potential antigenic candidates that might elicit an immune response for antibody production.

The selection of MAbs which were able to recognize a heat-stable epitope on the antigen was performed during the secondary screening of these hybridomas against protein extracts from both severely heat-treated (autoclaved) and raw blood of different animal species. Only those hybridomas that secreted MAbs that reacted with both the raw and autoclaved extracts were selected. This ensured that all the selected MAbs would bind to a true thermostable ligand on the antigen. The species specificity (mono, narrow, or broad specificity) of each of the selected MAbs was also determined. MAbs with narrow or broad species specificity could be useful for the future development of different immunoassays that offer more flexibility regarding species and binding selections.

Species Specificity of the MAbs. The species specificity of these MAbs was examined against raw, cooked, and autoclaved bovine, sheep, rabbit, horse, chicken, porcine, and turkey blood samples using indirect ELISA. **Table 1** summarizes the reactivity and reaction patterns of the eight selected MAbs. All the MAbs belong to subclass IgG1 and strongly react with cooked and autoclaved blood extracts of one or more of the species tested. These MAbs were categorized into three groups according to their immunoreactivities to heterologous blood. The first group includes three MAbs (Bb1B4, Bb2B11, Bb3D6) that displayed reactivity to blood proteins from two or more mammalian species of bovine, ovine, porcine, equine, or rabbit origin. The second group consists of four MAbs (Bb1H9, Bb6F10, Bb6G12, and Bb7F6) that reacted to blood proteins of ruminant species (bovine, ovine), while the third group comprises one MAb (Bb6E1) that recognized specifically bovine blood proteins. None of these MAbs reacted with poultry (chicken or turkey) blood proteins.

All MAbs reacted with autoclaved, cooked, and raw bovine blood, although the reaction against raw bovine blood was much weaker in the indirect ELISA. Because the antibodies were produced using autoclaved bovine blood as the immunogen, their reaction with bovine blood was expected. The weak reaction signal in raw blood samples compared to heat-treated samples was also expected, because in the indirect ELISA format the same amount (0.5 μg) of total soluble proteins is coated on each well of the microplate. The amount of thermostable antigens in the raw extract will be much less than that present in the heat-treated extracts because heat denatures and insolubilizes most of the soluble proteins present in the raw extract. Autoclaving was performed at 121 °C for 15 min at 1.2 bar and cooking was done at 100 °C for 15 min, representing a more severe heat treatment for autoclaved bovine blood compared to cooked bovine blood. The ability of these MAbs to react with raw, cooked, and autoclaved bovine blood indicates that the antigenic proteins are highly thermostable.

All of the MAbs except MAb Bb6E1 exhibited some cross reactivity with blood samples of other mammalian species. Antibodies recognize relatively small regions of antigens (epitopes) and hence can often find similar epitopes on closely related molecules in a sample, which forms the molecular basis

Table 1. Summary of the Distinctive Species-Specific Immunoreactivities of Monoclonal Antibodies (MAbs) to Proteins Extracted from Heat-Treated[a] and Nontreated Whole Blood of Different Animal Species[b] and Their Cross-Reactivity with Several Nonblood Proteins[c] Using Indirect ELISA

MAb (subclass)	species specificity			cross-reactivity with nonblood proteins tested
	autoclaved	cooked	raw	
Bb1B4 (IgG1)	equine ++++[d] porcine ++++ ovine ++++ bovine ++++	equine +++ porcine ++++ ovine ++++ bovine ++++	equine ++ porcine ++ ovine ++ bovine +++	–
Bb1H9 (IgG1)	bovine ++++ ovine ++++	bovine ++++ ovine ++++	bovine ++++ ovine ++++	–
Bb2B11 (IgG1)	bovine ++++ rabbit ++++	bovine ++ rabbit ++	bovine ++ rabbit +	BSA ++++
Bb3D6 (IgG1)	bovine +++ equine ++++ porcine +	bovine +++ equine ++++	bovine +	–
Bb6E1 (IgG 1)	bovine ++++	bovine ++++	bovine +	
Bb6F10 (IgG1)	bovine ++++ ovine +++	bovine ++ ovine ++	bovine +	
Bb6G12 (IgG1)	bovine ++ ovine ++++	bovine +++ ovine ++++	bovine +	
Bb7F6 (IgG1)	bovine ++ ovine ++++	bovine ++ ovine ++++	bovine +	

[a] Cooked at 100 °C for 15 min or autoclaved at 121 °C for 15 min. [b] Horse, pig, sheep, cattle, rabbit, chicken, and turkey. [c] Raw, cooked, and autoclaved meat proteins, bovine serum albumin, gelatin, and nonfat dry milk proteins. [d] +, weak reaction; ++, moderate reaction; +++, strong reaction; ++++, very strong reaction; –, negative reaction.

Table 2. Antigenic Components in the Blood Protein Extracts Probed by Each Monoclonal Antibody (MAb) Produced Using Western Blot[a]

MAb	autoclaved blood protein extracts and commercial bovine blood meals							estimated MW (kDa)
	Bb	Sb	Rb	Hb	Pb	Bbm	Bpm	
Bb1B4	++++[b]	++++	–	++++	++++	++	++	25
Bb1H9	+++	++	–	–	–	++	+++	10
Bb2B11	++	–	++	–	–	–	++	10
Bb3D6	++++	–	–	++++	+	++	++++	60
Bb6E1	++++	–	–	–	–	++++	++++	40
Bb6F10	++	+++	–	–	–	–	–	25
Bb6G12	++++	++++	–	–	–	++	+++	60
Bb7F6	++++	++++	–	–	–	++	+++	60

[a] Bb, bovine blood; Sb, ovine blood; Rb, rabbit blood; Hb, equine blood; Pb, porcine blood; Bbm, commercial bovine blood meal; Bpm, bovine plasma meal; MW, molecular weight of antigenic protein/peptide components. [b] +, weak reaction; ++, moderate reaction; +++, strong reaction; ++++, very strong reaction; –, negative reaction.

for species cross-reaction (*22*). This species cross-reactivity can be either desirable or undesirable, depending on the application. Immunoassays with a broad specificity are useful for screening for a group of targeted materials with a single test, while monospecific assays are desirable if specific species identification becomes necessary.

Cross-Reactivity with Nonblood Proteins. Commercially produced food products and feedstuffs also contain proteins from other sources, including animal tissues such as muscle or plant proteins such as soy protein. This section of the experiment was therefore designed to determine whether any of the MAbs cross-reacted with any nonblood proteins that are likely to be present in food and animal feed. The nonblood proteins were categorized into two groups, namely, nonflesh proteins (BSA, NFDM, soy protein, gelatin, and egg albumin) and flesh proteins (raw and heat-processed meat from bovine, sheep, rabbit, horse, chicken, turkey, and porcine species). BSA, a serum protein that is commonly used for blocking, was included under nonflesh proteins to ascertain if any of the antibodies cross-reacted with it because of its common use in the antibody dilution buffer. Egg albumin, soy protein, gelatin, and NFDM are common food or feed additives, with gelatin and NFDM also serving as possible choices for a blocking buffer. All the MAbs failed to cross-react with any of the nonflesh proteins tested except MAb Bb2B11, which strongly cross-reacted with BSA (**Table 1**). Consequently, any use of MAb Bb2B11 should take into account possible cross-reactions with BSA. None of these MAbs, however, showed any cross-reactions with muscle proteins.

Thermostable Proteins Recognized by the MAbs. The TSPs recognized in the blood protein of five mammalian species by each MAb were determined from Western blot analysis. To examine whether these MAbs could be useful in analyzing commercial blood feedstuff, a spray-dried bovine plasma sample and a bovine whole blood powder sample were also included in the analysis. **Table 2** summarizes the immunoblot results for all the MAbs developed. Overall, four antigenic TSPs in autoclaved blood samples were identified by the eight MAbs. MAbs Bb1H9 and Bb2B11 bind to a 10 kDa protein band in the autoclaved blood extract; MAbs Bb1B4 and Bb6F10

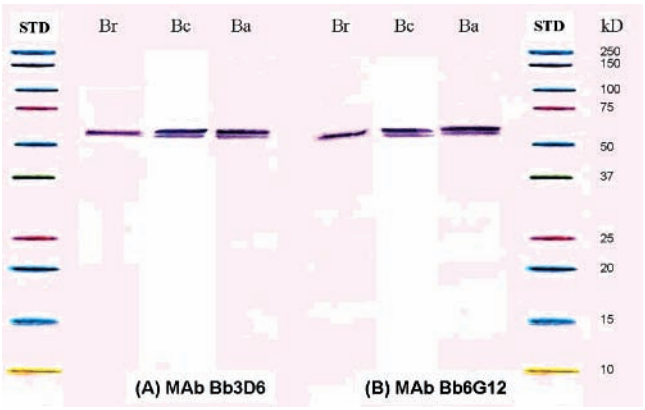

Figure 1. Western blot analysis of protein extracts from raw, cooked (100 °C, 15 min), and autoclaved (121 °C, 15 min) bovine blood with supernatants of (**A**) MAb Bb3D6 and (**B**) MAb Bb6G12. Protein samples (3 μg in 15 μL per lane) were loaded alongside the Precision Plus protein kaleidoscope prestained standards. Br: raw bovine blood; Bc: cooked bovine blood; Ba: autoclaved bovine blood; STD: Precision plus protein kaleidoscope standards.

recognize a 25 kDa protein band, MAbs Bb6E1 recognizes a 40 kDa protein band, and MAbs Bb3D6, Bb6G12, and Bb7F6 all bind to a 60 kDa protein band. These general species-specific reaction patterns closely match the results obtained from indirect ELISA (**Table 1**) that MAbs Bb1B4, Bb2B11, and Bb3D6 bind to blood proteins from two or more mammalian species of bovine, ovine, porcine, equine, or rabbit origin; MAbs Bb1H9, Bb6F10, Bb6G12, and Bb7F6 reacted to blood proteins of ruminant species (bovine, ovine), and MAb Bb6E1 recognized specifically bovine blood proteins. All of these MAbs except Bb6F10 reacted with protein extract of bovine blood meal.

The Western blot membrane of raw, cooked, and autoclaved bovine blood TSPs blotted by two representative MAbs, Bb3D6 and Bb6G12, are shown in **Figure 1** to demonstrate the identical antigenic protein bands probed by these two MAbs. Both MAbs Bb3D6 and Bb6G12 bind to a ~60 kDa antigenic protein in heat-treated (autoclaved and cooked) and raw bovine blood extracts. Pairing these two Mabs may be useful for a new sandwich ELISA if they exhibit noncompetitive binding. To determine if these two MAbs bind to the same or different epitopes on the 60 kDa antigenic protein, we performed an ELISA additivity test according to the method developed by Friguet et al. (*23*). The ELISA reading for the combined MAbs Bb3D6 and Bb6G12 in a well was much higher than the readings from each of the individual MAbs (additivity) indicating that these two MAbs bind at two distinct sites on the antigen molecules and exhibit noncompetitive binding. The 60 kDa antigenic protein is likely to be one of various reported plasma proteins with molecular weights of between 58 and 62 kDa. Candidate plasma proteins include α_1T-Glycoprotein (60 kDa), 4 S α_2-β_1-glycoprotein (60 kDa), C3 activator (60 kDa), histidine-rich 3.8 S α_2-glycoprotein (58.5 kDa), and thyroxine-binding globulin (60.7 kDa), which are present in normal serum at concentrations of 8, 0.02, 18, 9, and 1.5 mg%, respectively (*24*). Future research should be aimed at characterizing this thermostable protein as well as additional antigenic TSPs revealed by other MAbs.

Epitopes on antigen molecules may be formed by contiguous (sequential epitope) or noncontiguous (conformational epitope) amino acid sequences (*22*). The blood immunogen used to produce the MAbs in this study was subjected to severe heat treatment, which may unfold the TSPs and consequently change the secondary and tertiary structure of the TSPs by breaking hydrogen bonds and destroying other weak interactions. Heating at higher temperatures under pressure could further break covalent bonds between the amino acids in the protein's primary structure, thus degrading the protein into shorter polypeptides. The MAbs developed in this study are likely to recognize sequential epitopes rather than conformational epitopes since conformational epitopes are usually affected by heat treatment (*25*). This assumption is reinforced by the results of the Western blot analysis, where the bands were very strong for positive samples as was also the case for indirect ELISA. Western blot analysis involves the use of the denaturing agent SDS, which causes proteins to unfold or polypeptides to separate to assume an extended linear conformation (*26*).

Production of a panel of MAbs that recognize a single or several species-specific, blood-specific, and thermostable antigens in bovine blood would be useful for the detection of bovine blood in a mixture, regardless of the heat treatment conditions of the sample, with an immunoassay. Neither such MAbs nor information on thermostable bovine blood proteins is available in the current literature. This study reports the methods and procedures used to develop eight MAbs that are specific to blood of bovine origin or other mammalian or ruminant animal species and that bind to a thermostable ligand on an antigenic molecule (protein or peptide) in the blood. These MAbs thus can be used to detect the presence of blood of bovine, ruminant, or mammalian origin in a wide range of raw, cooked, or even sterilized samples.

This study has also explored the use of four thermostable blood proteins. Future characterization of these proteins would be of interest to scientists from biological, clinical, and other life science disciplines. Once a panel of MAbs has been developed, they can be employed in various formats of immunoassays (e.g., competitive or noncompetitive ELISA, lateral flow, immunosensors, etc.) individually or in a combined form for various purposes. The MAbs developed in this study will be useful for the detection of blood in food intended for human consumption or animal feed samples for labeling enforcement, forensic diagnosis, and further study of the properties of thermostable blood proteins.

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