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Novel Method for Detecting Bovine Immunoglobulin G in Dried Porcine Plasma as an Indicator of Bovine Plasma Contamination

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Current U.S. Food and Drug Administration regulations prohibit feeding of protein derived from mammalian tissue, excluding blood and blood products and any product that consists entirely of porcine or equine protein. A novel lateral flow immunoassay device has been developed that can quickly and qualitatively determine the presence of bovine immunoglobulin G (IgG), a major component in blood products, at very low levels (0.01% v/v). The device can be used to test for bovine IgG commingling in spray-dried porcine plasma used in the feed industry. Producers and consumers alike could use this device to verify product content at threshold levels.

KEYWORDS: Lateral flow immunoassay device; immunoglobulin G; spray-dried bovine plasma; spray-dried porcine plasma; ruminant feed; transmissible spongiform encephalopathy; bovine spongiform encephalopathy; variant Creutzfeldt−Jakob disease

INTRODUCTION

In recent years, the threat of neuralgic diseases acquired from animal feeds fed to foreign and domestic ruminant herds has become a serious issue for consumers, producers, and government regulatory agencies (*1*). Transmissible spongiform encephalopathies (TSE) such as scrapie, bovine spongiform encephalopathy (BSE), and its human form, new variant Creutzfeldt−Jakob disease (vCJD), have come to the forefront as serious economic and health risks worldwide (*2*). Under current U.S. Code of Federal Regulations (CFR), any ruminant feed derived from mammalian tissues is forbidden (*3*). This CFR, however, allows for the use of mammalian blood and blood products and any products having only protein of porcine or equine origin in ruminant feed products (*3*). Although bovine blood and plasma are currently acceptable for use, their future is uncertain due to changing attitudes of producers, blenders, and consumers who would like to have products that are "free" of bovine blood and plasma products (*4*).

Feed blenders and manufacturers usually acquire these blood and plasma products from renderers who process or transport products from both ruminants (cattle, buffalo, sheep, goats, deer, elk, and antelopes) and nonruminants (pigs, and horses (*4*). Because of this, care must be taken to ensure that cross-contamination does not take place in the blending or manufacturing process.

Due to the emergence of new information with regard to BSE and vCJD, the U.S. Food and Drug Administration (FDA) recently solicited comments and views as to whether the rule should be changed (*1*). Additionally, the FDA has solicited information on potential methods that could be used in detecting mammalian proteins in feed (*1*). Currently, there are only a limited number of commercially available assays that can successfully determine if these products contain protein from one or more species (*5*−*7*). Toward this end, a novel, lateral flow immunoassay device has been developed that can quickly and qualitatively determine the presence of bovine IgG, a major component in blood products, at very low levels in porcine plasma products used in the feed industry.

MATERIALS AND METHODS

Materials. Normal equine serum, porcine serum, and bovine serum used as immunoelectrophoresis (IEP) references and affinity-purified rabbit anti-goat IgG (H and L), 2.4 mg/mL total protein, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). IEP gels, buffers, and chambers were purchased from Sebia (Norcross, GA). Brilliant Blue R 250 and ACS grade tetrachloroauric(III) acid were purchased from Sigma Chemical Co. (St. Louis, MO). Protein G Sepharose 4 Fast Flow was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Actigel ALD-Superflow (6% agarose) and ALD coupling solution ($NaCNBH_3$) were purchased from Sterogene Bioseparations (Santa Clara, CA). Goat anti-equine IgG (H and L), goat anti-porcine IgG (H and L), and goat anti-bovine IgG (H and L) antibodies, normal swine serum, turbidimetric calibrators, and dilution buffer (0.05 M Tris, 0.3 M NaCl, 0.1% NaN3, 0.2% EDTA, pH 7.4) were obtained from Midland BioProducts Corp. (Boone, IA). Bovine and porcine blood samples were obtained by jugular venipuncture and collected into EDTA anticoagulant tubes. Blood was promptly centrifuged (15 min at 2500*g*) and pooled. The pooled plasma was stored at −20 °C and tested within 2 months. American Protein Corp. (Ames, IA) provided 14 spray-dried samples of porcine or bovine origin. The samples represented both blood products and flavor stocks (reduced bone hydrolysates). Minitan-S, Minitan tangential filter plates, and nitrocellulose membranes were purchased from Millipore Corp. (Bed-

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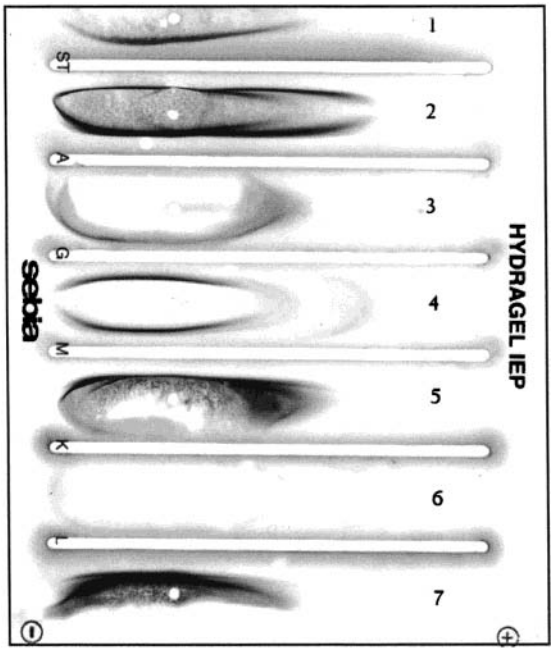

Figure 1. IEP showing interspecies cross-reactivity. Numbered wells contain (1 and 5) normal porcine serum (60 mg/mL total protein), (2, 4, and 6) normal equine serum (60 mg/mL total protein), and (3 and 7) normal bovine serum (60 mg/mL total protein). Troughs contain (ST and A) goat anti-equine IgG (63 mg/mL total protein), (G and M) goat anti-porcine IgG (85 mg/mL total protein), and (K and L) goat anti-bovine IgG (96 mg/mL total protein).

ford, MA). Polyvinyl-bound glass fiber was purchased from Whatman (Ann Arbor, MI). Wicking paper was purchased from Schleicher and Schuell, Inc. (Keene, NH). Solid phase plasma separation medium (sample pad) was purchased from Pall Corp. (Port Washington, NY). A BioJet Quanti3000 dispenser was purchased from BioDot (Irvine, CA). A Matrix 2210 universal laminator module was purchased from Kinematic Automation (Twain Harte, CA). Plastic cassette housings were purchased from Arista Biologicals Inc. (Bethlehem, PA).

Goat Anti-Bovine IgG Cross-Reactivity. Interspecies cross-reactivity was compared by IEP (**Figure 1**). The serum references (2.4 μL) were electrophoresed in a precast 1.1 g/dL agarose gel at 32 mA for 35 min at room temperature. Antiserum references and samples were added to the precut troughs (80 μL) in the gel and allowed to diffuse into the gel for 24 h at room temperature. The gel was stained with 0.125% Brilliant Blue R 250 in 25% (v/v) 2-propanol, 10% (v/v) acetic acid for 10 min. The gel was then destained in 25% (v/v) 2-propanol, 10% (v/v) acetic acid using several changes of this solution until the background cleared. Cross-reactivity to porcine IgG was removed from the goat anti-bovine IgG antibody by solid phase affinity chromatography adsorption.

Affinity Column Preparation. The affinity column was prepared from an IgG fraction of normal porcine serum by protein G affinity purification. Porcine serum was adsorbed over protein G Sepharose equilibrated with 0.02 M NaH_2PO_4, 0.15 M NaCl, pH 7.4. The unbound protein was washed from the column using the same buffer and discarded. The bound protein (porcine IgG) was eluted from the column with 0.1 M glycine−HCl, pH 2.8, and collected in 8 mL fractions. The eluted fractions were pooled and concentrated by tangential flow filtration to 21.4 mg/mL total protein. The affinity-purified porcine IgG (900 mg) was coupled to 50 g of 6% agarose using monoaldehyde linkage chemistry (*8*−*10*). The concentration of bound affinity-purified porcine IgG was 17.3 mg/g agarose as determined by monitoring (λ_{280nm}) the unbound supernatant.

Goat Anti-Bovine IgG Adsorption. Goat anti-bovine IgG (201 mg) was adsorbed over the affinity column equilibrated with 0.25 M Tris-HCl, 0.1 M glycine−HCl, 0.005 M glucose, pH 7.8. The unbound protein was washed from the column using the same buffer and collected in 8 mL fractions. The bound protein was eluted from the column with 0.1 M glycine−HCl, pH 2.8, and discarded. Unbound fractions were pooled and concentrated to 6 mg/mL total protein. The concentrated, unbound protein (goat anti-bovine IgG) was then re-adsorbed over the affinity column until no bound protein was observed (monitored at λ_{280nm}) upon elution. The resultant goat anti-bovine IgG was pooled and concentrated to a final concentration of 10.2 mg/mL total protein. Specificity of this adsorbed antibody was verified by IEP (**Figure 2**).

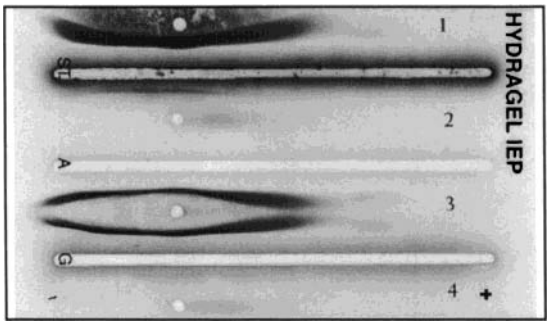

Figure 2. IEP showing intraspecies cross-reactivity removed. Numbered wells contaib (1 and 3) normal bovine serum (60 mg/mL total protein) and (2 and 4) normal porcine serum (60 mg/mL total protein). Troughs contain (ST) goat anti-bovine IgG with porcine reactivity (6.7 mg/mL total protein), (A) goat anti-bovine IgG without porcine reactivity (10.2 mg/mL total protein), and (G) goat anti-bovine IgG (96 mg/mL total protein).

Table 1. Cassette Results Using Standard Serum Preparations

	standard	% bovine plasma[a]	% porcine plasma[b]	cassette result
1	1.0%	1.000	99.000	positive
2	0.1%	0.100	99.900	positive
3	0.01%	0.010	99.990	positive
4	0.001%	0.001	99.999	negative
5	negative control	0	100	negative
6	positive control	100	0	positive

[a] Pooled from jugular venipuncture of calves. [b] Pooled from jugular venipuncture of piglets.

Gold Colloid Production and Antibody Conjugation. A 40 nm gold colloid was prepared from ACS grade tetrachloroauric[III] acid using standard colloid growth techniques (*11*−*14*). The adsorbed antibody was conjugated to the gold colloid at 10 μg/mL using general conjugation techniques for immunoglobulins (*11*, *13*).

Lateral Flow Device Development. The adsorbed goat anti-bovine IgG (as the test line reagent) and the gold conjugate were incorporated into a patented lateral flow immunoassay device format (*15*). A control line reagent (rabbit anti-goat IgG) was also incorporated into the device as a control mechanism. Both test and control line reagents were dialyzed into a buffer containing 0.015 M phosphate, 0.15 M sodium chloride, pH 7.4, and diluted to 1.0 and 1.2 mg/mL total protein, respectively. The reagents were applied to nitrocellulose at 0.75 μL/cm using a commercial capacity batch-spraying dispenser and dried for 30 min at 37 °C. The antibody/gold conjugate was applied to a polyvinyl-bound glass fiber sheet at 1.5 μL/cm. The dried nitrocellulose and antibody/gold conjugate were assembled using the laminator. A cellulose wicking pad and sample pad were added to complete the assembly. Strips were cut (4 mm) and housed in plastic cassette blanks.

Cassette Testing. The IgG concentration for each plasma pool was determined by turbidimetric assay (TIA) using commercial calibrators and methods (*6*). Standards were prepared by adding pooled bovine plasma to pooled porcine plasma at fixed volumes (**Table 1**). Using the dilution buffer, each standard was diluted 1:10. After 150 μL of each diluted solution had been added to the sample well of the cassette, it was left undisturbed on a level surface for 20 min. A red line was observed at the test line indicating a "positive" test for the presence of bovine IgG in the sample. If the sample did not contain bovine IgG, a red line was not observed at the test line (negative result). Regardless

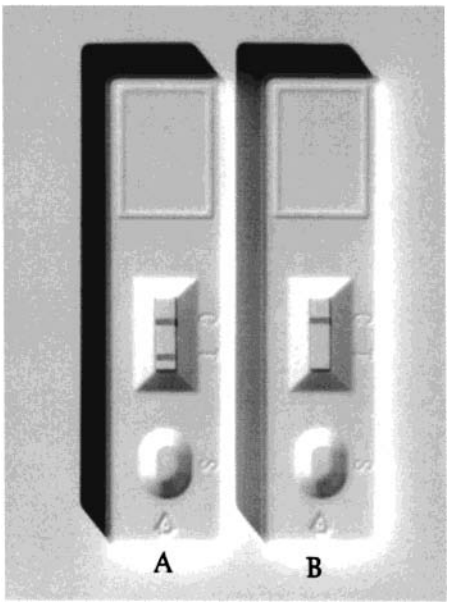

Figure 3. Lateral flow device interpretation: (A) single line at the test (T) position indicates a positive test for bovine IgG; (B) no line at the T position indicates a negative test for bovine IgG.

Table 2. Rehydrated Spray-Dried Product Testing Results

	sample composition	cassette result
1	pork plasma—random sample	positive
2	pork serum—random sample	positive
3	pork stock—random sample	positive
4	pork flavor—random sample	negative
5	"all pork" plasma—no known beef contamination	negative
6	beef whole blood—random sample	positive
7	beef plasma—random sample	positive
8	beef serum—random sample	positive
9	beef flavor—random sample	negative
10	beef stock—random sample	negative
11	"all beef" plasma—no known pork contamination	positive
12	bulk appetein—random sample	positive
13	mixed plasma 1—known contamination	positive
14	mixed plasma 2—known contamination	positive

Table 3. Interspecies Cross-Reactivity

	equine serum[a]	bovine serum[a]	porcine serum[a]
anti-equine IgG[b]	X		X
anti-porcine IgG[b]	X	X	X
anti-bovine IgG[b]		X	X

[a] X indicates interspecies cross-reactivity. [b] Of goat origin.

of the presence or absence of bovine IgG in the sample, a single red line developed at the control position ("C") of the cassette (**Figure 3**).

Fourteen spray-dried protein products of bovine and/or porcine origin were rehydrated, at 10% (w/v) with deionized water, for testing in the same method as the plasma standards (**Table 2**).

RESULTS AND DISCUSSION

Anti-Bovine IgG Cross-Reactivity and Removal by Adsorption. Cross-reactivity was observed by IEP (**Figure 1**) between goat anti-equine IgG and normal porcine serum (**Table 3**). Goat anti-porcine IgG cross-reacted with both normal equine serum and normal bovine serum, whereas goat anti-bovine IgG cross-reacted with normal porcine serum only (**Table 3**). After adsorption by affinity chromatography, the IEP indicated that bovine to porcine cross-reactivity was removed from the anti-bovine IgG antibody (**Figure 2**).

Cassette Testing. The six standards were prepared by introducing pooled bovine serum (bovine IgG, 1820.0 mg/dL) into pooled porcine serum (porcine IgG, 1171.3 mg/dL) at known dilutions (**Table 1**). These standards were then run in the cassettes and evaluated (**Figure 3**). Results of the standards in duplicate (**Table 1**) indicated that the device was able to qualitatively detect the presence of bovine IgG to 0.01% (v/v) in porcine serum but not below 0.001% (v/v).

Cassette Performance. Cassette testing (in duplicate) with spray-dried porcine products demonstrated visible control lines with all assays. Rehydrated spray-dried porcine products (plasma or serum) with low percentage of rehydrated spray-dried bovine products (plasma, serum, or whole blood) produced visible test lines. The device was able to differentiate between "all pork" (a negative result) samples and "all" or partial beef samples [a positive result (**Table 2**)] 100% of the time. The device tested with pork and beef flavor or stock product samples did not develop test lines [a negative result (**Table 2**)]. Pork and beef flavor or stock samples did not develop test lines (negative result), suggesting that no bovine plasma contamination existed in the bone hydrolysates or that processing made the IgG undetectable.

Conclusion. Many feed manufacturers use animal plasma or plasma products as a protein source for ruminant feed. FDA regulations prohibit feeding of protein derived from mammalian tissue, excluding blood and blood products and any products having only mammalian protein consisting entirely of porcine or equine protein (*1*). The device described here provides a rapid (20 min), qualitative detection of bovine IgG (plasma) contamination in porcine plasma products used for feed. With a detection level of 0.01% (v/v), the device is more sensitive than a turbidimetric immunoassay (TIA) method of bovine IgG quantitation, which is accurate to 2.5% (v/v) (*6*). The cassette format provides a disposable test, with minimal sample preparation, laboratory equipment, expertise to operate, and storage requirements (3-year shelf life at room temperature) and may be performed at the field site. With simple, fast, and reliable data, producers and consumers alike can use this assay device to detect bovine IgG content at threshold levels.

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