FOOD BIOLOGICAL CONTAMINANTS

Detection of Pork in Heat-Processed Meat Products by Monoclonal Antibody-Based ELISA

FUR-CHI CHEN and Y-H. PEGGY HSIEH [1]
Auburn University, Department of Nutrition and Food Science, Auburn, AL 36849-5606

An enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody to a porcine thermal-stable muscle protein was developed for detection of pork in cooked meat products. The assay specifically detects porcine skeletal muscle, but not cardiac muscle, smooth muscle, blood, and nonmuscle organs. No cross-reactivity was observed with common food proteins. Validity of the assay was evaluated with laboratory formulated and commercial meat samples. The detection limit was determined as 0.5% (w/w) pork in heterologous meat mixtures. Overall, intra- and inter-assay coefficients of variation were 5.8 and 7.9%, respectively. The accuracy in analyzing market samples was 100% as verified by product labeling and confirmed by a commercial polycolonal antibody test kit.

Analytical methods for detection of meat species adulteration are imperative for quality assurance and regulatory purposes. Identification of species origin in heat-processed meat products requires a different approach from methods used for raw meats, which detect heat labile serum proteins. Enzyme-linked immunosorbent assay (ELISA) protocols of the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) for identification of cooked meats use polyclonal antibodies (PAbs) against heat-resistant muscle glycoproteins (1, 2). Antisera to heat-treated muscle proteins of different preparations have also been developed to identify cooked meats (3–6). Because of the heterogenous nature of antisera, potential problems are associated with the use of PAbs. The preparation of species-specific antisera requires immunoaffinity adsorption of cross-reactivity, which is a costly and time-consuming procedure. Moreover, variations in specificity and affinity between batches of antisera are the major concerns in developing standardized procedures (7).

The value of using monoclonal antibody (MAb) in immunoassays is due to its homogeneous nature and biologically well-defined characteristics. The use of a MAb-based ELISA could reduce the cost of analysis and provide a standardized assay for the increased need for routine analyses (8). Although several MAbs against muscle proteins have been developed for identification of raw meat (8–13), only a few have been produced for detection of heat-processed meats (14–16). We previously reported the development of 4 MAbs (5H9, 5H8, 2F2, 8A4) to a porcine thermal-stable muscle protein (14). The MAbs differentiated pork from other common meat species (beef, horse, lamb, deer, chicken, turkey, and duck) in both raw and cooked products. Application of these MAbs in ELISA for detection of heat-processed meat products would provide an attractive alternative to currently used PAbs.

Assays for detection of species adulteration must be able to work in a complex and variable matrix (7, 17). Hence, the MAb-based ELISA for meat species identification must overcome potential cross-reactivity with other meat species and food additives. For validation of a developed assay, the laboratory-formulated meat mixtures might not represent the vast variety of meat products on the market. Because it is impractical to test individual ingredients and conditions for potential interferences with numerous model systems, trials must be conducted on a variety of meat products to assess the validity of a developed assay. The present study was undertaken to optimize an ELISA using MAb 5H9 for detection of pork in heat-processed meat products and to evaluate assay validity in testing commercial meat products.

Experimental

Materials

Porcine organs and tissues (liver, heart, brain, spleen, skin, blood, kidney, tongue) and whole pork ham and beef round were obtained from Auburn University Meat Laboratory. Pork chitterlings and stomach, and nonfat dry milk were purchased from a local retail market. Glycerol, thimerosal, sodium chloride, sodium phosphate, citric acid, biotinamidocaproate *N*-hydroxysuccinimide (NHS-CA-biotin), 2,2′-azino-di-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), streptavidin–horseradish peroxidase (HRP) conjugate, and 30% hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Goat–anti-mouse IgG–HRP conjugate was obtained form Bio-Rad Laboratories (Hercules, CA). Soy protein isolate was obtained from A.E. Staley Manufacturing Co. (Decatur, IL). Tween 20, gelatin, bovine serum albumin (BSA),

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[1] Author to whom correspondence should be addressed.

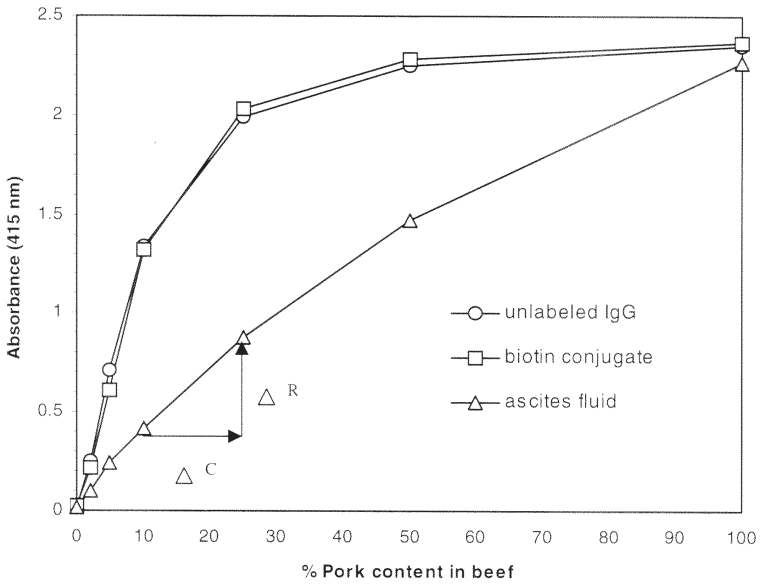

Figure 1. Dose response curves of indirect ELISA using ascites fluid, purified IgG, and biotin conjugate of MAb 5H9 as immunoreagents. Values shown are means of 5 replicate measurements. ΔR = changes in absorbance; ΔC = changes in pork content.

egg albumin, and polyvinylchloride microtiter plates (Costar, Cambridge, MA) were purchased from Fisher Scientific (Pittsburgh, PA).

Purification of Monoclonal Antibody

Production of MAb 5H9 to porcine thermal-stable muscle protein has been described previously (14). MAb 5H9 (IgG1) was purified from ascites fluid by a Protein A affinity column using the Econo low pressure chromatography system (Bio-Rad). The purified antibody was dialyzed against 0.01M phosphate-buffered saline (PBS, pH 7.0) overnight at 4°C with several changes of the dialysis buffer. Concentration of IgG in the final preparation was determined by UV spectrophotometer at 280 nm, and the purity of antibody was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Concentration of antibody was then adjusted to 2 mg IgG/mL with PBS. The biotin-conjugated MAb 5H9 was prepared by the methods of Guesdon et al. (18) using NHS-CA-biotin. The purified IgG and biotin conjugate were stored at –20°C with addition of 50% glycerol and 0.05% thimerosal.

Laboratory-Prepared Meat Samples

Meats from fresh pork ham and beef round were used to formulate laboratory samples. Lean muscles were prepared by trimming off connective tissue and visible fat. Muscle tissues were ground twice and mixed thoroughly. Meats from different species, and porcine organs and tissues were processed separately. The grinder and utensils were cleaned carefully when different samples were prepared to prevent cross contamination. Ground meats were then divided into portions of ca 300 g each and stored in sealed freezer bags at –80°C. The meats were completely thawed at room temperature for 3 h before use.

Quality control samples with known adulteration levels of 50, 25, and 2% (w/w) pork in beef were formulated by combining preweighed ground pork and beef into 10 g portions and placed in glass tubes (2.5×15 cm) with threaded caps. Unadulterated pork and beef samples were processed in a similar manner. The tubes were then heated in boiling water for 15 min and cooled immediately in cold water. To each tube, 20 mL 0.5M NaCl in 0.01M sodium phosphate buffer (pH 7.0) was added, and the samples were homogenized with a Brinkmann Polytron Homogenizer Model PT 10/35 (Brinkmann Instrument Co., Westbury, NY). The homogenates were set undisturbed at room temperature for 1 h, and then centrifuged at $2000 \times g$ for 30 min. The supernatants were filtered through Whatman No. 1 filter paper, (Fisher Scientific, Pittsburgh, PA) and protein concentration of the filtrate was determined by the method of Bradford (19) using BSA as the standard. Aliquots of sample extracts were stored in glass vials at –80°C until use.

Calibration standards were prepared daily by adjusting pure pork and beef extracts to the same protein concentration and mixed at different ratios to yield adulteration levels of 0.5,1, 2, 5, 10, 25, 50, and 100% (v/v) of pork in beef. Porcine organs and food proteins (10% w/v, suspension of egg albumin, BSA, gelatin, nonfat dry milk, and soy protein isolate in 0.5M NaCl containing 0.01M sodium phosphate, pH 7.0) for testing cross-reactivity were heated in boiling water for 15 min as described above, and the same procedure as for extraction of meat samples was followed.

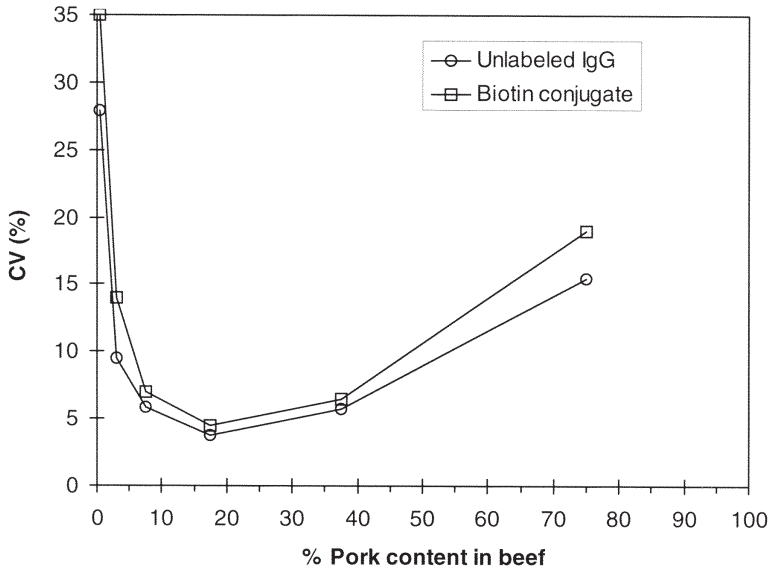

Figure 2. Precision profile of indirect ELISA using purified IgG and biotin conjugate of MAb 5H9. The coefficients of variation of determination (CV, %) for each data point were calculated from 10 intra-assay replications.

Commercial Meat Samples

Forty-five retail samples were collected from a local grocery store. All samples were precooked or canned meat products of various categories, including ham, sausage, franks, bologna, salami, spread, and luncheon meat. Content of these products consisted of pork, beef, chicken, or turkey as the only meat ingredients or a combination of 2 or more meat species. Sample portions (10 g) were placed in glass tubes (2.5×15 cm) and heated in boiling water for 15 min prior to analysis. Extraction followed the procedures described above, and extracts were analyzed for the presence of pork by indirect ELISA. For verification of the results, all commercial samples were also tested with a cooked meat speciation kit (ELISA-TEK, ELISA Technologists, Inc., Alachua, FL) following manufacturer's instruction.

Indirect ELISA

The dilution of meat extracts, concentration of antibodies, incubation time, and temperature for performing ELISA were optimized for maximum sensitivity and enhanced detectability while maintaining precision. Two detection systems, unlabeled IgG and biotin conjugated MAb 5H9, were tested. Optimal conditions were determined by analyzing the precision profiles (20) obtained from various changes of assay conditions. Sensitivity ($\Delta R/\Delta C$) was determined from dose response curves as changes in ELISA response (ΔR) related to changes of pork content (ΔC). Detection limit was defined as the lowest pork content at which the response is equal to 3 standard deviations (SDs) above the response of zero-dose sample (e.g., pure beef). The precision profile was plotted as pork content (C) against coefficient of variation (CV) of determination, $CV = [SD/(\Delta R/\Delta C)]/C$. The reproducibility of the assay was assessed by comparing precision profiles of different experimental conditions.

The assay procedures with optimized condition are described as follows: Meat extracts were diluted in 0.06M carbonate buffer (pH 9.6) to a final protein concentration of 0.04 mg/mL. Wells of the microtiter plate were filled with 100 μL diluted meat extracts; the plate was then placed in a thermostatic incubator at 37°C for 1 h. After incubation, the plate was washed 3 times with 0.01M phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) using a microplate washer (Bio-Rad, Model 1250). Each well of the plate was filled with 200 μL blocking buffer (1% BSA in PBS) and incubated for 30 min at 37°C. After another washing step, 100 μL 1:2000 diluted MAb 5H9 (0.5 μg IgG/mL) in antibody buffer (1% BSA in PBS-T) was added to each well and incubated at 37°C for 1 h. The plate was washed again and 100 μL 1:2500 diluted goat–anti-mouse IgG peroxidase conjugate in antibody buffer was added to each well. After 1 h incubation at 37°C , the plate was washed 5 times with PBS-T, and 100 μL enzyme substrate (22 mg ABTS and 15 μL 30% hydrogen peroxide in 100 mL 0.1M phosphate–citrate buffer, pH 4.0) was added to each well. Color development was processed for 30 min at 37°C. Enzyme reaction was stopped by adding 100 μL 0.1M citric acid, and absorbance was measured at 415 nm using a microplate reader (Bio-Rad, Model 450). When the biotin conjugated MAb 5H9 was used in the indirect ELISA, an appropriate diluted (1:1000) streptavidin–HRP conjugate was used instead of the goat–anti-mouse IgG–HRP conjugate; other assay conditions were kept the same.

Calibration standards and quality control samples were included in each plate. Dose response curves were generated for each assay, and regression analysis was performed to fit the sigmoidal curve using the Hill equation (21),

Table 1. Cross-reactivity of MAb 5H9 with porcine organs and tissues and common food proteins, as determined by indirect ELISA

Samples	Absorbance at 415 nm[a]
Skeletal muscle[b]	2.351 (0.009)
Tongue	2.339 (0.005)
Heart	0.001 (0.001)
Stomach	0.012 (0.004)
Chitterlings	0.022 (0.002)
Skin	0.025 (0.015)
Kidney	0.032 (0.007)
Liver	0.015 (0.006)
Spleen	0.013 (0.002)
Brain	0.018 (0.002)
Blood[c]	0.021 (0.012)
Serum	0.023 (0.005)
Bovine serum albumin	0.001 (0.001)
Egg albumin	0.016 (0.008)
Dry milk powder	0.012 (0.007)
Soy protein isolate	0.009 (0.004)
Gelatin	0.012 (0.003)

[a] Mean of 4 replications (standard deviation).
[b] Skeletal muscle from pork ham.
[c] Serum-free blood clot.

$Y = [aX^b / (c^b + X^b)]$, where Y and X are absorbance and adulteration level (%), respectively; a, b, and c are 3 parameters determined from the regression curve. The adulteration levels of control samples were calculated from a linear plot of log X against log (Y/[a – Y]), which transforms the Hill equation into $\log X = \log c + b^{-1} \log (Y/[a - Y])$. Analytical data of control samples were recorded on Shewhart control charts (22).

Results and Discussion

Optimization of ELISA

Optimal conditions of the assay were obtained with 0.04 mg protein/mL diluted meat extract in carbonate buffer, 1:2000 dilution of MAb 5H9, 1:2500 dilution of goat–anti mouse IgG–HRP (or 1:1000 of streptavidin–HRP, when biotin-conjugated MAb 5H9 was used), 1 h at 37°C for antibody incubation, and 30 min at 37°C for substrate reaction. It was expected that use of biotin-conjugated MAb 5H9 would result in higher sensitivity and detectability than the unlabeled one. However, similar dose response curves were observed for both experimental conditions (Figure 1). Assay sensitivity ($\Delta R/\Delta C$) was greatly improved at the low-dose region (adulteration level < 25%) with purified IgG or biotin-conjugated MAb 5H9 as compared with ascites fluid. The increased sensitivity at low dose would enable a clear differentiation of samples with lower adulteration levels from negative samples.

The precision profile of ELISA showed that the CV of the determinations was <10% at the adulteration level, 2–50% for unlabeled IgG, and 5–45% for biotin conjugate MAb 5H9 (Figure 2). Imprecision increased rapidly at pork contents higher or lower than this range. Both labeled and unlabeled MAb 5H9 yielded satisfactory sensitivity, whereas detectability was better for unlabeled MAb 5H9. Detection limit of the assay was determined as 0.5 and 0.8% (w/w) of pork in beef mixtures using unlabeled antibody and biotin conjugate, respectively. In addition, the precision profile indicated less variation of unlabeled IgG. Unlabeled antibody, therefore, was used for the rest of the experiments.

ELISA Performance

The cross-reactivity of ELISA to porcine organs and tissues as well as to common food proteins was examined. The assay differentiated pork skeletal muscle from cardiac muscle, smooth muscle, and nonmuscle organs (Table 1). No cross reaction was observed for all the organs and tissues except pig tongues. Because of the similarity among voluntary muscles, both tongue and skeletal muscle exhibited the same reactivity. Skeletal muscle specificity is a unique characteristic of this assay, which is different from other published methods where cross-reactivity to organs, cardiac muscle, or smooth muscle tissues has been observed (4, 6, 9, 13). Food proteins including milk proteins, egg albumin, soy proteins, and gelatin did not interfere with detection. This is important because such proteins are frequently used as additives in commercially prepared meat products.

Reproducibility of assay was further evaluated using quality control samples at low, medium, and high adulteration levels (2, 25, and 50% pork in beef) over 4 weeks. Standard curves using Hill plot were applied to determine adulteration levels in control samples; regression coefficients (R^2) >0.995 were normally obtained from daily analysis. Reproducibility of the assay was calculated from analytical data of control samples. Variability was lowest for the medium level control (intra-assay CV = 3.1% and inter-assay CV = 5.6%), followed by high level control (intra-assay CV = 6.4% and inter-assay CV = 8.7%) and low level control (intra-assay CV = 8.0% and inter-assay CV = 9.4%). Overall intra- and inter-assay CVs for control samples over this period were 5.5 and 7.9%, respectively. Based on the magnitude of SDs and CVs of determinations for control samples with 3 adulteration levels for 18 individual runs (Figure 3), the assay system clearly demonstrated highly reproducible results.

Analysis of Commercial Samples

The market samples for trial testing included a variety of pre cooked meat products, including frank, sausage, salami, sliced meat, and canned meat. The cut-off value for a negative sample was set at the 1% adulteration level, which, in terms of absorbancy units, must be <0.1 after subtracting 3 SDs from the mean absorbance. Among the 45 samples tested, 18 contained pork and the remaining 27 were unambiguously determined as negative (Table 2). With traditional agar–gel immunodiffusion, the detection limit of the assay was usually

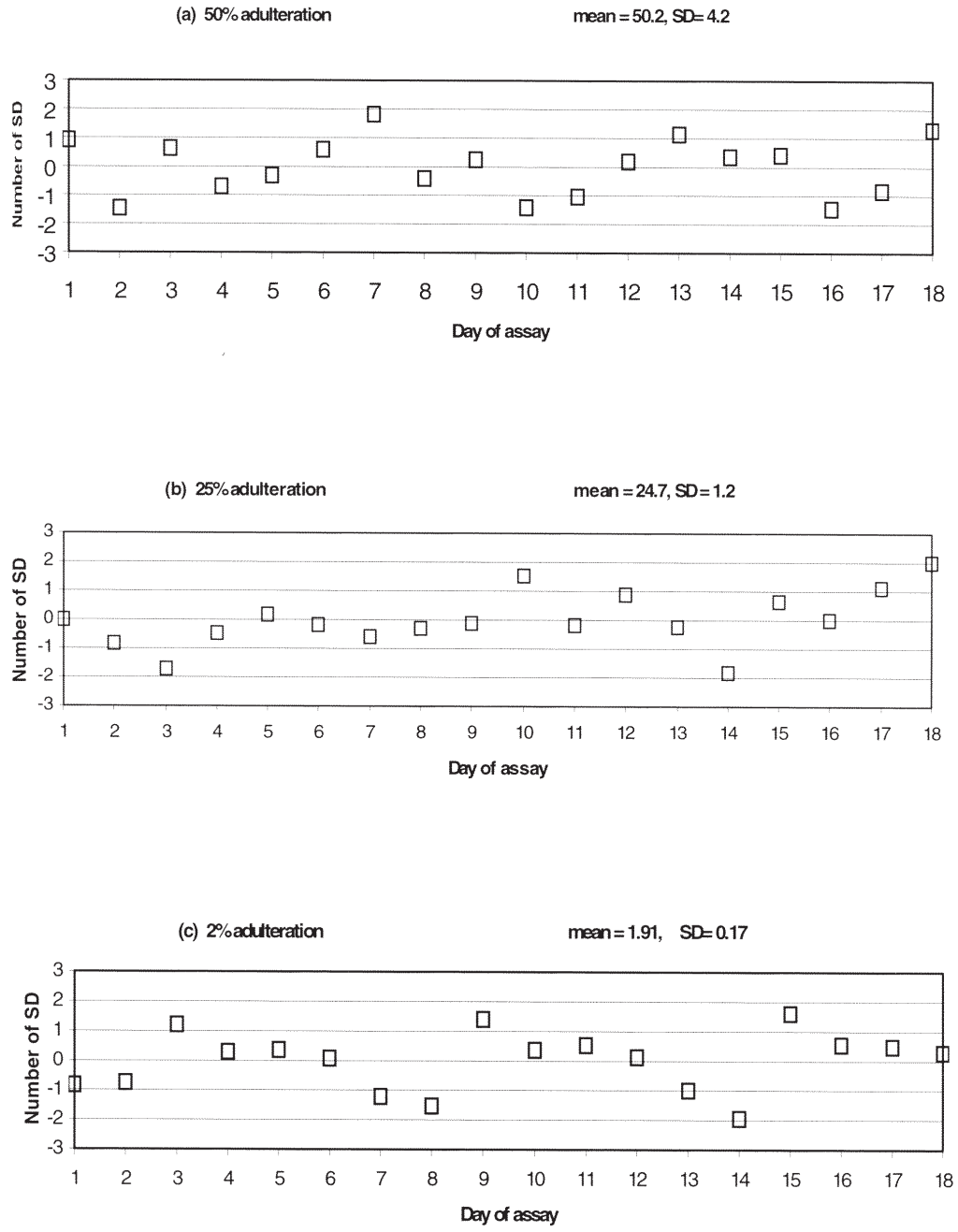

Figure 3. Shewhart charts of 3 control samples at (a) 50% pork, (b) 25%, and (c) 2% pork content for 18 individual tests on different days. Each data point represents the mean of 3 replicate samples.

at the 5–10% levels (17). Judging violative samples was, therefore, confined by the minimum level that the method could achieve. With the enhanced detectability of ELISA, very low levels of adulterant can be detected in meat samples. For practical purposes, a cut-off value must be set for judging violations. Hsieh et al. (23, 24) applied 1% as the criterion for reporting the violation rate of species adulteration in official meat samples, because adulteration below such level would not yield an economic incentive. Although assays that detect even lower levels of adulteration (0.1% or less) could be developed, an assay capable of detecting down to 1% adulteration seems adequate for routine analysis.

As confirmation, all of the samples were also analyzed by a commercial ELISA kit and compared with the species content labeled on the ingredient lists (Table 2). Results of the MAb-based ELISA and the commercial test kits agreed with the product labeling. The assay developed in the present study was proved 100% accurate for detection of pork in market samples, and the presence of various additives did not interfere with the detection. As shown in a previous study (24),

Table 2. Detection of pork in commercial meat samples

Sample	Product name	Species content[a]	MAb-ELISA[b]	PAb-Test Kit[b]
1	*Franks*	B, P, T	0.751 (0.025)	0.949 (0.054)
2	Turkey smoked sausage	T	0.003 (0.005)	0.004 (0.006)
3	Beef smoked sausage	B	0.006 (0.005)	0.058 (0.016)
4	Beef franks	B	0.016 (0.009)	0.010 (0.008)
5	*Polska kielbasa*	B, P, T	2.353 (0.028)	0.983 (0.026)
6	*Smoked sausage*	P	2.336 (0.014)	1.044 (0.053)
7	*Smoked sausage*	B, P, T	1.275 (0.031)	0.912 (0.027)
8	Beef bologna	B	0.010 (0.021)	0.008 (0.002)
9	*Bologna*	B, P, C	2.064 (0.029)	0.871 (0.055)
10	*SPAM*	P	2.247 (0.053)	0.879 (0.006)
11	Corned beef	B	0.007 (0.007)	0.015 (0.004)
12	Corned beef	B	0.016 (0.012)	0.013 (0.001)
13	*Deviled ham spread*	P	2.277 (0.006)	0.984 (0.056)
14	Chicken spread	C	0.021 (0.007)	0.010 (0.012)
15	Roast beef spread	B	0.016 (0.003)	0.005 (0.006)
16	*Bologna*	P, C	2.218 (0.040)	0.834 (0.042)
17	Beef bologna	B	0.025 (0.005)	0.016 (0.006)
18	*Smoked cotto salami*	B, P, Ph, C	2.226 (0.014)	0.856 (0.021)
19	Beef cotto salami	B, Bh	0.017 (0.011)	0.026 (0.005)
20	*Cotto salami*	B, Bh, P, C	2.194 (0.040)	0.912 (0.006)
21	Chicken frankfurters	C	0.011 (0.008)	0.012 (0.004)
22	Jumbo beef franks	B	0.024 (0.018)	0.003 (0.001)
23	*Jumbos franks*	B, P, C	0.991 (0.014)	0.910 (0.019)
24	Jumbo beef franks	B	0.014 (0.013)	0.001 (0.002)
25	Turkey jumbo franks	T	0.005 (0.008)	0.007 (0.002)
26	Roast beef	B	0.010 (0.005)	0.017 (0.002)
27	Smoked turkey breast	T	0.021 (0.007)	0.010 (0.004)
28	*Cooked ham*	P	2.389 (0.012)	1.118 (0.055)
29	Smoked turkey breast	T	0.023 (0.006)	0.012 (0.005)
30	*Smoked ham*	P	2.356 (0.030)	1.017 (0.042)
31	Smoked turkey breast	T	0.012 (0.013)	0.001 (0.002)
32	Roasted cured beef	B	0.028 (0.012)	0.001 (0.001)
33	Smoked turkey ham	T	0.003 (0.005)	0.001 (0.001)
34	Corned beef	B	0.011 (0.005)	0.001 (0.001)
35	*Smoked ham*	P	2.343 (0.025)	1.034 (0.023)
36	Smoked chicken	C	0.019 (0.009)	0.001 (0.001)
37	Smoked beef	B	0.010 (0.001)	0.004 (0.001)
38	Smoked turkey	T	0.006 (0.001)	0.001 (0.002)
39	*Smoked ham*	P	2.383 (0.005)	1.138 (0.003)
40	*Cocktail smoked sausage*	P	2.280 (0.011)	0.878 (0.037)
41	Beef cocktail smoked sausage	B	0.007 (0.004)	0.006 (0.002)
42	Beef sausage links	B	0.009 (0.004)	0.016 (0.004)
43	*Sausage links*	P	2.232 (0.023)	0.809 (0.005)
44	*Smoked sausage*	B, P	2.348 (0.025)	0.846 (0.013)
45	Beef smoked sausage	B	0.013 (0.007)	0.020 (0.004)
46	Control (pure beef)	B	0.009 (0.006)	0.007 (0.001)

Table 2. (*continued*)

Sample	Product name	Species content[a]	MAb-ELISA[b]	PAb-Test Kit[b]
47	*Control (2% pork in beef)*	B, P	0.229 (0.012)	0.595 (0.008)
48	*Control (25% pork in beef)*	B, P	2.131 (0.026)	0.746 (0.018)
49	*Control (50% pork in beef)*	B, P	2.272 (0.015)	0.748 (0.023)
50	*Control (pure pork)*	P	2.353 (0.003)	0.748 (0.033)

[a] Species content from product labeling. B = beef, Bh = beef heart, P = pork, Ph = pork heart, C = chicken, T = turkey.

[b] Results are presented as mean absorbance of 3 replicated measurements (SD). Products in italics indicate pork-positive samples.

name-brand products had a lower incidence of adulteration than market-made products; therefore, commercial samples of name-brand manufacturers were selected for this study to avoid complications in the interpretation of results. All samples were correctly labeled in terms of pork content, but the compliance of labeling with respect to other meat species was not determined. As estimated from the calibration curve, all the positive samples contained >5% pork, with 4 containing <25% pork. Because the content of pork in these products could not be verified by labeling information or analytical methods, quantitative data were not reported. In addition, variations in sample composition, which affect recovery of antigen from samples, e.g., pH, salt content, and cooking temperature, need to be considered in interpretation of quantitative results.

Conclusions

To our knowledge, the assay developed in the present study is the first MAb-based assay reported in the literature that can detect heat-treated porcine meat without cross-reactivity with other species. The MAb-based ELISA reliably detected pork in various commercial meat products. Analytical validity, including specificity, sensitivity, detectability, and reproducibility suggested that the ELISA developed was suitable not only for qualitative detection, but also as a potential method for quantifying adulteration levels. Further investigation will evaluate the validity of the assay for quantitative determination of adulteration levels in cooked meat products.

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