Evaluation of a Rapid PCR-Based Method for the Detection of Animal Material

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MS 05-185: Received 18 April 2005/Accepted 29 June 2005

ABSTRACT

A rapid PCR-based analytical method for detection of animal-derived materials in complete feed was developed. Using a commercially available DNA forensic kit for the extraction of DNA from animal feed, a sensitive method was developed that was capable of detecting as little as 0.03% bovine meat and bone meal in complete feed in under 8 h of total assay time. The reduction in assay time was accomplished by reducing the DNA extraction time to 2 h and using the simpler cleanup procedure of the kit. Assay sensitivity can be increased to 0.006% by increasing the DNA extraction time to an overnight incubation of approximately 16 h. Examination of dairy feed samples containing either bovine meat and bone meal, porcine meat and bone meal, or lamb meal at a level of 0.1% (wt/wt basis) suggested that this method may be suitable for regulatory uses. The adoption of this commercially available kit for use with animal feeds yields an assay that is quicker and simpler to perform than a previously validated assay for the detection of animal proteins in animal feed.

Detection of processed animal proteins (PAP) in complete feed by PCR-based methods has been demonstrated by numerous laboratories (4). One PCR-based method has passed a method-validation trial (6). For that procedure, DNA is extracted onto silica from the feed matrix, which has been first solubilized in a guanidine thiocyanate–based buffer (6). Although the method is robust, it is labor intensive and requires a minimum of 24 h to analyze a sample. The analyst also must prepare all the reagents. The labor requirements of this method limit the number of samples a single analyst can process in a day, which increases the cost of the assay. Another limitation of this method is the silica. Improper preparation of this key reagent results in a failure to properly or consistently extract DNA from the feed matrix, necessitating quality-control measures to be enacted for each batch of silica to ensure functionality (3).

One potential solution to these issues would be to use commercially available kits to extract the DNA. One such kit is the DNA forensic kit (Invitrogen, Gaithersburg, Md.). This kit was designed to extract DNA from a wide variety of forensic samples and is, in fact, a commercial variant of the silica–guanidine thiocyanate (GSCN) method. Accordingly, we examined this kit to determine its capability to extract animal DNA from animal feed. The results of these analyses would determine their suitability for use in regulatory laboratories focusing on the detection of animal-derived materials in animal feed. As part of this process, efforts were also initiated to determine means to reduce the total sample analysis time.

MATERIALS AND METHODS

Reagents. The silica, ethanol, acetone, guanidine thiocyanate, EDTA, Trizma-HCl, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, Mo.). The Tris-EDTA and Tris-borate-EDTA buffers, along with the SuperMix, were obtained from Invitrogen (Gaithersburg, Md.).

Animal feed. Dairy feed containing various levels of rendered animal-derived protein were prepared in the feed mill at the Office of Research as previously described (7). The feed consisted of cracked corn (30%), oats (35%), soybean meal (26%), dicalcium phosphate (2.5%), soybean oil (1%), dried molasses (2.5%), salt (NaCl; 1.5%) (AgWay, Gettysburg, Pa.), and dairy premix #4 (1.5%; C.S. Akey, Inc., Lewisburg, Ohio). Various amounts of either bovine meat and bone meal (BMBM; generously provided by the Excel Corp., Wyalusing, Pa.), porcine meat and bone meal (PMBM; generously provided by IPB, Logangsport, Ind.), or lamb meal (LM; purchased on the open market; Australian in origin) were mixed with 15 kg of the above feed to produce feed at varying levels of fortification (0.006 to 2%). Control feed (nonfortified; true negative sample) was obtained from the same batch of feed used to prepare animal feed–fortified feed samples. The BMBM and PMBM samples were processed at temperatures ranging from 125 to 131°C under atmospheric conditions for approximately 30 min. Animal protein fortification was performed by mixing the animal material with the ground feed in a Hobart mixer. The feed was mixed for 15 min at room temperature (approximately 22°C). Aliquots of finished feed were placed into Whirl-Pak bags (Nasco, Ft. Atkinson, Wis.) and stored at room temperature.

DNA extraction: silica–GSCN method. The silica–GSCN method has been previously described (7) and validated (6). Briefly, 0.5 g of complete dairy feed containing varying levels of BMBM was mixed with 4.5 ml of extraction buffer (5 M guanidine thiocyanate; 0.02 M EDTA, pH 8.0; 0.05 M Trizma-HCl, pH 6.4; 1.3% Triton X-100) and allowed to incubate overnight (14 to 16 h) at 60°C. The supernatant was placed in a new tube following centrifugation (4,200 × g, 10 min) to remove undissolved material. Five hundred microliters of the clarified supernatant was mixed with 0.5 ml of extraction buffer and 0.04 ml of silica sus-
The silica was prepared according to Boom et al. (1). After a 10 min incubation at room temperature, the silica was precipitated by centrifugation (13,000 x g, 2 min), then washed three times with wash buffer (extraction buffer minus EDTA and Triton X-100), two times with 70% ethanol, and one time with acetone. Each wash was accomplished by resuspending the silica pellet, followed by centrifugation (13,000 x g, 2 min). The DNA was extracted from the silica with 0.5 ml of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Five microliters of the DNA-containing solution was used for the PCR reaction.

DNA extraction: DNA forensic kit method. The Invirob Forensic Kit I (Invitex, distributed by Syngen, Inc., San Diego, Calif.) was used according to the manufacturer’s instructions for “DNA Isolation from Bone or Ancient Bone Material.” Briefly, DNA was extracted from approximately 250 to 270 mg of bone or bone powder with 1,000 μl of lysis buffer D and 200 μl of bone lysis enhancer (kit supplied). The material was incubated overnight at 60°C. After centrifugation (9,300 x g, 10 s), the DNA in the resulting supernatant was extracted with the carrier suspension. Impurities that coprecipitate with the carrier suspension were removed by washing the carrier suspension (three times) with wash buffer. The spent wash buffer was removed by centrifugation (9,300 x g, 10 s). The DNA was eluted from the carrier suspension with the elution buffer (50 μl). After each wash step (6), the carrier suspension was pelleted by centrifugation (16,000 x g, 2 min). Following each centrifugation (6), the DNA in the supernatant was removed to a new micro test tube.

Effective DNA extraction from animal feed required some modifications to this base method. DNA from animal feed was extracted by this approach by substituting approximately 250 to 270 mg of feed for the bone or bone powder. Because high-grain-content feed samples resulted in supernatant samples that could be viscous after the overnight incubation step, an additional 200 μl of lysis buffer D was added to reduce the viscosity. After the carrier suspension was first centrifuged following incubation with the supernatant from the extracted feed, there typically were impurities that could not be removed with just the kit wash buffer. Therefore, the first wash of the carrier suspension was performed with 1,000 μl of lysis buffer D, followed by two wash steps with the wash buffer. To improve the removal of the carrier suspension following DNA extraction with elution buffer, a third centrifugation step was added.

PCR and PCR primers. The PCR primers specific for bovine, porcine, and ovine mitochondrial DNA have previously been detailed (5), as has the “universal” primer which simultaneously detects DNA derived from bovine, equine, ovine, caprine, cervid, and porcine materials (5). The PCR primers L8127 and H8372 (bovine specific), L8049 and H8273 (universal), and PL15 and PH281 (porcine specific) are as previously described and were prepared by Invitrogen.

Positive and negative control samples were included in all PCR analyses. Positive controls were species-specific DNA (bovine, ovine, or porcine DNA) isolated from peripheral blood, while the negative control was distilled water. Positive control DNA was isolated from freshly drawn heparinized blood with the Promega Wizard Genomic DNA Purification Kit (Madison, Wis.). This method isolates both genomic and mitochondrial DNA.

Each PCR reaction tube contained 5 μl of extracted DNA and 45 μl of a PCR master mix prepared by mixing 44 μl of SuperMix (Invitrogen) and 0.5 μl of each primer (forward and reverse). PCR amplification was accomplished by 29 cycles of denaturing at 94°C for 1 min (the first denaturing step is performed for 2 min) and annealing for 30 s, followed by extension at 72°C for 1 min. The last cycle used denaturing at 94°C for 1 min, annealing for 30 s, and extension at 72°C for 10 min. The annealing temperature was 48°C for primers L8049 and H8273, 58°C for primers L8127 and H8372, and 58°C for primers PL15 and PH281.

The PCR products were identified by size with a 100 bp ladder. After completion of the PCR reaction, 12-μl samples (10 μl of PCR product mixed with 2 μl of 5× gel-loading buffer, Embi Tec, San Diego, Calif.) were subjected to gel electrophoresis with a 2% agarose gel prestained ethidium bromide and visualized with UV light (Embi Tec). The PCR products from the bovine and universal primers result in amplicons 271 bp in length, while the product of the porcine primers result in an amplicon 212 bp in length.

The relative amounts of PCR amplicon were spectrophotometrically determined with a Bio-Rad Gel Doc 2000 gel documentation system (Hercules, Calif.). The relative absorbance values were entered into Excel 2000 (Microsoft, Redmond, Wash.). The results were graphed with SigmaPlot 8.0 (Chicago, Ill.).

RESULTS

The DNA forensic kit is a commercial variant of the silica-GSCN method previously validated for use with animal feed to detect BMBM (6). Therefore, the initial task was to determine if the kit could be adapted for use with animal feed by using the maximal amount of feed possible within the constraints imposed by the quantities of reagents supplied in the kit. Through trial and error, it was ascertained that a maximum sample size of 250 to 270 mg of grain-based feed, in a volume of 1,200 μl (1,000 μl of lysis buffer D and 200 μl of bone lysis enhancer), could be analyzed. This yielded 100 to 300 μl of DNA-containing supernatant when examining complete feed and up to 1,000 μl when extracting DNA from pure meat meals. Samples with a high grain content are hydrated by the buffer, resulting in a supernatant solution more viscous than the starting buffer. Conversely, supernatant from samples with minimal grain content, such as meat and bone meal, had a viscosity similar to that of the starting buffer. Sample viscosity had the potential to impact DNA adsorption onto the carrier suspension by preventing uniform dispersal of the carrier suspension. The greater viscosity from grain-based samples did not permit complete mixing of the carrier suspension. This problem was eliminated by the addition of 200 μl of lysis buffer D to the supernatant. While this approach resulted in a uniform dispersal of the carrier suspension, it did not reduce the amount of contaminating material that coprecipitated with the carrier suspension. This material was not removed by using just the wash buffer supplied with the kit. The addition of a wash step with the lysis buffer D prior to using the kit’s wash buffer did result in the elimination of this contaminant. These efforts resulted in the development of an assay capable of examining for the presence or absence of rendered animal proteins in feed.

To ascertain the sensitivity of the DNA forensic kit, feed samples with varying levels of BMBM (0.06 to 2%, wt/wt) were incubated overnight by either the silica-GSCN method or DNA forensic kit method. Analysis of the PCR
results obtained by the DNA forensic kit demonstrated a similar level of sensitivity (0.06%; Fig. 1B, bottom) as the silica-GSCN method, although the amplicon signal strength was not as strong as that of amplicons from DNA prepared by the silica-GSCN method (Fig. 1A, top).

The results for both the silica-GSCN method and DNA forensic kit indicated comparable levels of sensitivity following overnight incubation (14 to 16 h). The question became whether overnight incubation was needed to reach that level of sensitivity. As an initial starting point, a 2-h incubation period was selected; this incubation time would result in an assay of a single feed sample to be completed within a single workday. As anticipated, the reduction in the incubation time reduced the strength of the resulting amplicon signals when using either the silica-GSCN method (Fig. 1A, bottom) or the DNA forensic kit (Fig. 1B, bottom). The sensitivity of the silica-GSCN method following a 2-h incubation period easily achieved a level of 0.125%, with the amplicon for the 0.06% level visible. In contrast, the amplicons generated with the DNA forensic kit (Fig. 1B, bottom) were much less intense following a 2-h incubation period compared with the silica-GSCN method. Again, the amplicon for the 0.06% level of BMBM was visible, though faint.

To better understand the relationship between assay sensitivity and incubation time, the DNA from dairy feed samples containing varying levels of BMBM (2 to 0.007% wt/wt) were incubated at 60°C for varying time periods (15 min, 30 min, 1 h, 2 h, 20 h), and the resulting DNA was amplified by PCR with bovine-specific primers. The data points represent the lowest level of BMBM that could be detected at each incubation time.

Overnight incubation resulted in the ability to detect as little as 0.007%, a level comparable to that of the silica-GSCN method (5). In contrast, a 2-h incubation resulted in an assay sensitivity of 0.03%; decreasing the incubation time to just 15 min decreased the assay sensitivity to just 0.125% BMBM.

In order to ascertain the suitability of this method for regulatory use, multiple dairy feed samples containing either 0.1% BMBM, 0.1% PMBM, or 0.1% LM were analyzed following a 2-h incubation using the DNA forensic kit. Twenty-eight dairy feed samples containing BMBM (Fig. 3A) were examined by using the bovine-specific PCR primers. All 28 samples yielded a PCR amplicon with an average relative absorbance value of 1,012 ± 53 (standard error of the mean [SEM]; range 500 to 1,626). Thirty dairy feed samples containing LM (Fig. 3B) resulted in 29 samples yielding a PCR amplicon with an average relative absorbance of 2,236 ± 99 (SEM; range 1,454 to 3,188). Thirty PMBM-containing dairy feed samples were analyzed by using the porcine-specific PCR primers. These samples yielded 28 PCR amplicons with an average relative absorbance of 733 ± 59 (SEM; range 243 to 1,868).
The DNA forensic kit was found to be almost as sensitive for the isolation of animal-derived DNA in feed as the more robust silica-GSCN method. The DNA forensic kit method was capable of consistently detecting as little as 0.1% animal protein in complete animal feed, the level of sensitivity to which the silica-GSCN method was validated (6). This level of sensitivity is also consistent with that attained by feed microscopy, which is the official method currently used by the U.S. Food and Drug Administration (FDA) and the European Commission (2).

The DNA forensic kit demonstrated fairly consistent results when multiple analyses of the same type(s) of feed were performed. Analysis of dairy feed containing 0.1% BMBM with the bovine-specific primers demonstrated just a threefold range in the intensity of the resulting amplicon signals, with all 28 samples yielding a PCR amplicon. Likewise, dairy feed containing LM demonstrated just a twofold range in amplicon signal intensity when examined with the universal primers, with 29 out of 30 samples yielding an amplicon. The results obtained by using BMBM or LM are consistent with the acceptance criteria established for the validation trial of the silica-GSCN method (6). In contrast to these results were those for the porcine primers when used in combination with dairy feed fortified with PMBM. In this instance, there was a 7.5-fold range in amplicon signal intensity, with only 28 out of 30 samples yielding an amplicon. These results could be interpreted as suggesting that there are differences in PMBM that result in less than optimal DNA extraction with this kit. However, the BMBM- and LM-containing feed samples were isolated over a 2-day period, with no more than 15 test samples each day. All samples were extracted by a single analyst.

The experience gathered up to that point suggested that it might be possible for a single analyst to process more than 20 samples per day. The 30 PMBM-containing feed samples were all isolated on a single day by the same analyst. Although there were no obvious problems encountered during the day of isolation, there may be subtle issues associated with processing such a large number of samples to be processed on a single day by a single analyst. This suggests a practical limit on the number of samples a single analyst can or should process in a single workday.

The DNA forensic kit is, in fact, capable of detecting even lower amounts of PAP. If the extraction period is extended from the 2 h to overnight (approximately 16 h) the sensitivity can be increased to 0.007%. This level of sensitivity is identical to that reported for the silica-GSCN method (4). Although the most sensitive methods are usually of desirable quality for regulatory methods, this level of sensitivity may not be needed, especially in light of the sensitivity of feed microscopy (0.1%), the FDA’s official procedure. A confirmatory method, such as PCR, needs to be at least as sensitive as the determinative method (feed microscopy) and be able to confirm the animal materials.

DISCUSSION

The current method of amplicon detection relies on gel photography and photo-interpretation of the resulting image. Such reliance represents a potential weakness in this and similar methods of identification. When the range of test samples being analyzed included highly positive ones, the resulting images were often a compromise between an image that focused on the higher-intensity amplicons versus an image that focused on the lower-intensity amplicons. Reliance on the former results in the lower-intensity bands being faint to undetectable, while the latter results in images in which the highly positive samples obscured surrounding sample lanes. This points to the need to have the positive controls near the expected intensity of the unknown test sample(s). It also illustrates the need for detection and quantitation systems that rely less on art or the experience of the analyst.

This observation, however, does not detract from the utility of the DNA forensic kit. The present study, while presented as a unified method, is, in fact, two different “methods”: a DNA extraction method followed by a PCR method for DNA detection. The PCR method used in conjunction with the DNA forensic kit is the same one used in the successful validation of a PCR-based approach to the detection of BMBM in complete feed. Thus, photographic analyses of PCR reactions can yield a method capable of being used for regulatory purposes.

This method will most likely be used as a confirmatory method by the FDA. Microscopy is the official method of the FDA because of the regulation being enforced. The FDA’s feed ban, 21 CFR 589.2000, prohibits feeding mammalian-derived proteins to ruminants, with some notable exemptions (pure porcine, pure equine, blood). In contrast, the Import Alerts issued by the U.S. Department of Agriculture prohibits the importation of any PAP, unless a valid import permit is held by the importer. The only method capable of simultaneously detecting all possible PAP is microscopic examination. However, PCR has the discriminatory power to confirm or refute microscopy results. Microscopy can distinguish between PAP derived from avian, aquatic, or mammalian origin. It cannot distinguish between poultry (exempt) and pigeon (filth) or porcine (exempt) and bovine (prohibited).

This PCR-based method could be used as a primary screening tool by other regulatory agencies, such as state...
laboratories that are concerned with enforcement of the FDA’s feed ban. The attributes of this current method (daily sample capacity, sensitivity, specificity, PCR primer availability) are amenable to its use as a screening tool.

One unexpected benefit of this off-the-shelf kit is that eliminating the need to constantly prepare reagents and perform quality control analyses on these reagents results in a net savings of both analyst time and assay costs. Part of this savings also lies in the fact that the DNA carrier suspension (i.e., the silica) will have a level of assay consistency between laboratories not always possible when using “homemade” silica suspensions. Improper preparation of the silica suspension was one of the principal reasons that the European Commission validation trial of the original PCR-based method failed (2); half of the participating laboratories could not properly prepare the silica.

In conclusion, the DNA forensic kit represents an off-the-shelf technology that has the very real potential to improve the reliability and reproducibility of the analyses performed by laboratories using PCR to assess compliance with the feed ban.

REFERENCES