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Development of a Multiplex Real-Time PCR Assay for the Detection of Ruminant DNA

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ABSTRACT

The U.S. Food and Drug Administration (FDA) has previously validated a real-time PCR-based assay that is currently being used by the FDA and several state laboratories as the official screening method. Due to several shortcomings to the assay, a multiplex real-time PCR assay (MRTA) to detect three ruminant species (bovine, caprine, and ovine) was developed using a lyophilized bead design. The assay contained two primer or probe sets: a ''ruminant'' set to detect bovine-, caprine-, and ovine-derived materials and a second set to serve as an internal PCR control, formatted using a lyophilized bead design. Performance of the assay was evaluated against stringent acceptance criteria developed by the FDA's Center for Veterinary Medicine's Office of Research. The MRTA for the detection of ruminant DNA passed the stringent acceptance criteria for specificity, sensitivity, and selectivity. The assay met sensitivity and reproducibility requirements by detecting 30 of 30 complete feed samples fortified with meals at 0.1% (wt/wt) rendered material from each of the three ruminant species. The MRTA demonstrated 100% selectivity (0.0% false positives) for negative controls throughout the assessment period. The assay showed ruggedness in both sample selection and reagent preparation. Second and third analyst trials confirmed the quality of the written standard operating procedure with consistency of results. An external laboratory participating in a peer-verification trial demonstrated 100% specificity in identifying bovine meat and bone meal, while exhibiting a 0.03% rate of false positives. The assay demonstrated equal levels of sensitivity and reproducibility compared with the FDA's current validated real-time PCR assay. The assay detected three prohibited species in less than 1.5 h of total assay time, a significant improvement over the current real-time assay. These results demonstrated this assay's suitability for routine regulatory use both as a primary screening tool and as a confirmatory test.

Transmissible spongiform encephalopathies are neurodegenerative disorders that affect several mammalian species, including humans. In humans, transmissible spongiform encephalopathies typically occur sporadically, with the source of infection usually not identified *(2)*. One notable exception is a variant form of Creutzfeldt-Jakob disease (vCJD), which is believed to have resulted from the foodborne transmission of bovine spongiform encephalopathy (BSE) *(3, 10)*. In order to prevent the establishment and spread of BSE in U.S. cattle populations, the U.S. Food and Drug Administration (FDA), which has regulatory jurisdiction over all animal feed and feed ingredients, introduced regulation 21 CFR 589.2000 ''Animal Proteins Prohibited in Ruminant Feed'' in 1997 *(1)*. The regulation established a flexible system of controls designed to ensure that ruminant feed does not contain animal protein derived from prohibited tissues and to encourage innovation in such controls. The FDA has supported the regulation in the past using microscopy to detect mammalian, avian, or aquatic meat meals in finished feeds and feed components. Feed microscopy, however, is unable to differentiate materials derived from exempt species (porcine and equine) from materials derived from prohibited species (such as bovine, ovine, caprine, and cervine). Traditional PCR was used to distinguish the specific mammalian species.

Numerous laboratories have developed PCR assays to detect processed animal proteins in complete feed *(4, 5, 8, 9)*. The FDA Center for Veterinary Medicine, Office of Research (CVM OR) has previously validated both traditional and real-time PCR–based assays *(6, 7, 11)*. The previous real-time PCR–based assay was developed using a commercial DNA extraction kit coupled with simplex real-time PCR *(11)*. This assay was more efficient than the traditional PCR assays while still accurately determining whether there were prohibited materials, such as bovine, ovine, or caprine products, in the test sample. It is currently being used by the FDA and several state laboratories as the official screening method. However, there are several shortcomings to this real-time PCR assay. This method

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requires three separate PCR reactions to evaluate whether a sample contains bovine, ovine, or caprine materials. Since it was projected that most test samples would contain no prohibited material, the results of the real-time PCR assay could not distinguish between a true-negative sample and a failed PCR reaction. Finally, the assay needed to have a higher throughput if the assay was to achieve the full savings in cost and time typical for PCR-based assays.

This study was initiated to simplify the detection of prohibited material through the development of a multiplex real-time PCR assay (MRTA). The MRTA consists of a lyophilized bead format containing one primer or probe set to detect DNA specific for three prohibited ruminants in meals and feeds and a second primer or probe set to serve as an internal PCR control. The MRTA was then evaluated according to the FDA CVM OR's stringent criteria. To meet these criteria, the MRTA had to demonstrate specificity (detecting only target materials), sensitivity (identifying true positives), and selectivity (identifying true negatives). Criteria were set using a statistical approach, with success requiring a 90% probability of achieving the correct response within a 95% confidence interval. A minimum detection level of 0.1% meat and bone meal (MBM) was also required, consistent with the sensitivity of the validated PCR-based assay currently used as an aid in enforcement of the FDA's feed ban *(11)*.

MATERIALS AND METHODS

Animal DNA. Animal DNA was purchased from Zyagen (San Diego, CA).

Preparation of animal meals. The bovine meat and bone meal (BMBM), porcine meat and bone meal (PMBM), and ovine meat and bone meal (OMBM) samples were processed at temperatures ranging from 125 to 131°C under atmospheric conditions, for approximately 30 min. The BMBM was generously provided by the Excel Corp. (Wichita, KS), and OMBM was purchased from a commercial vendor. Caprine meat meal (CMM) was prepared from authentic goat meat obtained from an outside source and verified as authentic using species-specific PCR analysis. The CMM was prepared by a multistep purification process to remove fat and other unwanted materials. The meat meal was autoclaved twice for 15 min at 121°C and 15 psi, using a 10-min dry cycle (10 psi). The European Union (EU)–processed bovine meat meal (BMM), bovine bone meal (BBM), porcine meat meal (PMM), and porcine bone meal (PBM), referred to as "VLA MBM control material," was a generous gift of Mr. Scott Reaney (Veterinary Laboratory Agency, DEFRA, UK). The BMM and BBM were mixed in a 60:40 ratio to approximate meat and bone meal. This material was subjected to a chloroform wash and then allowed to air dry before being finely ground.

Test feed preparation. Dairy feed containing animal-derived proteins was prepared on-site at FDA CVM OR *(6, 7, 11)*. The dairy feed consisted of cracked corn (30%), oats (35%), soybean meal (26%), dicalcium phosphate (2.5%), dairy premix #4 (1.5%; C.S. Akey Inc., Lewisburg, OH), soybean oil (1%), dried molasses (2.5%), and salt (1.5%). Each animal meal was mixed with 5 kg of dairy feed to achieve a 0.1% (wt/wt) level of fortification. Animal protein fortification was performed by mixing the animal material with the ground feed in a Hobart mixer for 15 min at room temperature to ensure homogeneity. Feed was divided into single-use aliquots and stored at room temperature. Thirty samples of 0.1% fortified material were prepared for each species, as well as one control sample containing no rendered material.

Feed DNA extraction. Feed extractions were performed using the ChargeSwitch gDNA Rendered Meat Purification Kit (Invitrogen Corp., Carlsbad, CA) *(9)*. Extractions were performed using the method outlined by Yancy et al. *(11)*. Briefly, two 250-mg samples of feed were weighed out. Pure animal meals required only one 250-mg sample of meal. ChargeSwitch Lysis buffer and 10% sodium dodecyl sulfate were added to each sample. After a 5-min incubation at 95°C, precipitation buffer was added to each sample. Samples were then placed on ice for 5 min and were centrifuged to pellet debris. The supernatant from each of the two initial samples was transferred to a single tube. The supernatant from the pure animal meal samples was not combined. Next, the 10% detergent solution and ChargeSwitch beads were added to each sample and incubated briefly at room temperature to allow the beads to bind to the DNA. Samples were then placed on a MagnaRack, where the supernatant was removed. Samples were washed three times using the provided wash buffer and the MagnaRack. The samples were eluted into a clean tube using the provided elution buffer, and the ChargeSwitch beads were discarded. The purified DNA was stored at −20°C.

PCR primers and probes. A primer and probe set was developed by BioGX, Inc. (Birmingham, AL) to detect ruminant material from bovine, sheep, and goat. The ruminant-specific primers and probes were developed using published sequence information (accession no.: bovine DQ487094, sheep EU834864, goat EU623453, and deer FJ392291) available through the National Center for Biotechnology Information (NCBI) database, using Allele ID software (Premier Biosoft, Palo Alto, CA) to align the sequences to yield the ruminant-specific primers and probes. The proprietary primers and probes were developed to amplify DNA from the prohibited ruminants in meals and feed. The custom-designed TaqMan primer and probe set targets a 145-bp region, identified in silico as specific for the prohibited ruminant types according to sequence information available in the NCBI database. The proprietary primer and probe set report amplification when DNA of the prohibited ruminant types is present. The primer and probe set was paired with a second primer and probe amplification control, used to ensure that the PCR reaction was successful. The two primer and probe sets were combined in a lyophilized bead. The lyophilized material consisted of excipients for lyophilization, an internal amplification control (IAC) template, and an optimal concentration of oligonucleotides and reporter probes for amplification and detection of ruminant targets and IAC. Primers and probes used in this study will be commercially available (BioGX product no. 204-001).

Multiplex PCR protocol. Assay mix was prepared using two OmniMix HS Lyophilized PCR Master Mix beads (Cephid, Sunnyvale, CA), one MRTA bead, and 100 μl of water per four samples tested. The multiplex assays were conducted using the Smart Cycler II (Cepheid, Sunnyvale, CA). Thermocycling conditions were set to 50°C for 3 min, 95°C for 5 min, followed by an amplification program of 40 cycles at 95°C for 15 s, 60°C for 30 s (optics on), 60°C for 30 s (optics off). The FCTC25 dye set was used for fluorescence monitoring. A positive result for ruminant DNA (true positive or false positive) consisted of a sample having a cycle threshold (Ct) value in the FAM channel within 40 cycles. The IAC reports in the Cy5 channel and is used

TABLE 1. *Specificity of MRTA toward purified animal DNA*[a]

Species	Primer/probe set[b]	
	Ruminant	IAC
Cow (cattle)	+	−
Bison (ox)	+	−
Goat	+	−
Sheep	+	−
Deer	−	+
Bison (*Bison bison*)	−	+
Cat	−	+
Catfish	−	+
Elk	−	+
Dog	−	+
Duck	−	+
Ferret	−	+
Fly	−	+
Goose	−	+
Guinea pig	−	+
Hamster	−	+
Horse	−	+
Human	−	+
Monkey (baboon)	−	+
Monkey (rhesus)	−	+
Mouse	−	+
Pig	−	+
Pigeon	−	+
Quail	−	+
Rabbit	−	+
Rat	−	+
Salmon		−
Turkey	−	+

[a] Pooled (n = DNA from two to four animals) DNA samples were analyzed three times. The samples were amplified with the multiplex real-time PCR assay (MRTA) using a Smart Cycler II.

[b] IAC, internal amplification control; +, an amplification signal was detected for that primer and probe combination; −, there were no amplification signals detected for that primer and probe combination.

to measure sample inhibition of the real-time PCR and to help mitigate false-negative reporting. In the absence of a ruminant signal, the IAC reported at a Ct value of 35. Failure of the IAC to amplify at all or by a Ct of 35 indicated that inhibitory compounds were present in the sample and that further processing or clean up (a recommended 1:10 to 1:20 dilution of the sample) was required. If the sample was positive on the Cy5 channel at 35 Ct for the IAC and there was no reporting in the FAM channel, then the sample was "negative for ruminant DNA."

RESULTS

Specificity toward target and nontarget animal DNA. The MRTA tested for cross-reactivity with other nontargeted animal DNA templates. The specificity was evaluated against a panel of pooled DNA (two to four animals per pool) from tissue from 28 animal species: bison, cow, ox, goat, cat, catfish, deer, elk, dog, duck, ferret, fly, goose, guinea pig, hamster, horse, human, baboon, rhesus monkey, mouse, sheep, pig, pigeon, quail, rabbit, rat, salmon, and turkey. Each DNA pool contained the DNA from a single species. The assay only detected cow, goat, and sheep DNA. No cross-reactivity was observed in the remaining nontarget animal samples, indicating that the assay accurately identified the targeted species (Table 1).

TABLE 2. *Results of multiplex with feed*[a]

	Primer/probe set[b]	
	Ruminant	IAC
Feed (0.1%)		
Control	0/30 (0.0)	30/30 (100.0)
PMBM	0/30 (0.0)	30/30 (100.0)
BMBM	30/30 (100.0)	0/30 (0.0)
OMBM	30/30 (100.0)	0/30 (0.0)
CMM	30/30 (100.0)	4/30 (13.3)
Success rate by primer		
True-negative rate	100%	100%
True-positive rate	100%	100%
False-positive rate	0.0%	4.4%
False-negative rate	0.0%	0.0%

[a] IAC, internal amplification control; PMBM, porcine meat and bone meal–fortified feed; BMBM, bovine meat and bone meal–fortified feed; OMBM, ovine meat and bone meal–fortified feed; CMM, caprine meat meal–fortified feed. Dairy feed was fortified with PMBM, BMBM, OMBM, or CMM at 0.1% (wt/wt). DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

[b] Results for positive feed samples (BMBM, OMBM, and CMM) are expressed as the number of positive results/number of samples and accuracy (percentage of positive results in parentheses) for samples. Results for negative controls (control and PMBM containing feed) indicate the false-positive rate.

Sensitivity and selectivity assessment. In order to ascertain the suitability of this assay for regulatory use, dairy feed samples containing 0.1% BMBM, OMBM, CMM, PMBM, and a control feed containing no animal materials, were analyzed. The assay passed the sensitivity acceptance criteria at the 0.1% fortification level by detecting all of the samples containing the bovine, ovine, and caprine materials but did not yield a positive result when assaying feed containing nontarget PMBM (Table 2). The assay demonstrated that the ruminant primer exhibited complete sensitivity for the target material (100% sensitivity: 100% true positives, 0% false negatives). In addition, the assay was selectivity, as it did not yield any false positives for samples fortified with PMBM or for control samples (100% selectivity: 0% false positives, 100% true negatives).

Mixed feed samples. To determine if the presence of different combinations of mammalian proteins affects performance of the assay, extracts of DNA derived from different fortified animal meals were admixed and then subjected to the MRTA. Ten samples were prepared by mixing 1 μl of each feed extraction in various combinations of BMBM, OMBM, CMM, PMBM, and deer meat meal (DM). All samples containing target material BMBM,

TABLE 3. *Mixed feed samples*[a]

Proteins	Primer/probe set[b]	
	Ruminant	IAC
BMBM + PMBM	+	−
BMBM + OMBM	+	−
BMBM + CMM	+	−
BMBM + DM	+	−
OMBM + PMBM	+	−
OMBM + CMM	+	−
CMM + PMBM	+	−
CMM + DM	+	−
BMBM + OMBM + CMM	+	−
PMBM + DM	−	+

[a] Ten samples were prepared by mixing 1 μl of DNA from each feed extraction in different combinations. DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II. IAC, internal amplification control; BMBM, bovine meat and bone meal–fortified feed; PMBM, porcine meat and bone meal–fortified feed; OMBM, ovine meat and bone meal–fortified feed; CMM, caprine meat meal–fortified feed; DM, deer meat meal–fortified feed.

[b] +, an amplification signal was detected for that primer and probe combination; −, there were no amplification signals detected for that primer and probe combination.

OMBM, and CMM tested positive (Table 3). The addition of either PMBM or DM did not affect the capacity of the MRTA to detect the target species. The presence of multiple target DNA species also did not affect the capacity of the assay to yield a positive response.

Ruggedness in reagent preparation. In order to determine whether using an incorrect amount of water was detrimental to the acceptable performance of the assay, MRTA mastermix was prepared with ±40% of the recommended amount of water in 10-μl increments (60, 70, 80, 90, 100, 110, 120, 130, and 140 μl; 100 μl is the optimal amount of water). Feed samples containing 0.1% BMBM were then analyzed by PCR. The assay successfully detected 9 of 9 samples. Variances using the incorrect amounts of water (±40 μl) were determined to not impact the performance of the assay (Table 4).

TABLE 4. *Ruggedness testing*[a]

Water (μl)	Ruminant	Ct
60	+[b]	21.05
70	+	21.18
80	+	21.10
90	+	21.36
100 (baseline)	+	21.86
110	+	22.29
120	+	23.43
130	+	24.42
140	+	25.96

[a] Bovine meat and bone meal (0.1%) DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit. The multiplex real-time PCR assay (MRTA) mix was prepared with ±40% of the recommended amount of water in 10-μl increments (60, 70, 80, 90, 100, 110, 120, 130, and 140 μl) amplified using a Smart Cycler II. Ct, cycle threshold.

[b] +, an amplification signal was detected for that primer and probe combination.

TABLE 5. *Ruggedness in feed sample selection*[a]

Selection	Ruminant[b]
Large	3/3
Small	3/3

[a] The assay was evaluated for sample selection. Large was selected randomly and allowed to contain larger pieces; small was selected more carefully to include only the smallest particles from the complete feed. Bovine meat and bone meal (0.1%, wt/wt) DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

[b] Number of positive results/number of samples.

Ruggedness in feed sample selection. To ascertain the effect of different sampling strategies on the assay, 12 250-mg samples of feed were collected from feed fortified with 0.1% BMBM. Six of the samples were selected randomly and were allowed to contain larger particles, including cracked corn and whole pieces of wheat. The second set of samples was carefully selected to contain only small particles. The samples were extracted and analyzed. No difference in assay performance was seen between the two methods of sample collection (Table 5).

Second and third analyst trial. To identify possible deficiencies in the method write-up before its migration into regulatory laboratories, 15 DNA extractions from control feed and feeds fortified with 0.1% (wt/wt) BMBM, OMBM, or CMM were analyzed. A second and third analyst were selected and blinded as to the identity of the sample contents and study design. The assay met the acceptance criteria for selectivity, sensitivity, and specificity (Table 6). Each analyst was able to meet the acceptance criteria for selectivity and sensitivity for this assay, achieving 100% selectivity (detection of true negatives) and 100% sensitivity (detection of true positives). Neither analyst had any false-negative or false-positive results.

EU feed samples. To determine whether the assay could successfully detect DNA from rendered EU material, 30 dairy feed samples containing 0.1% EU-rendered BMBM were analyzed. The assay passed the sensitivity acceptance criteria at the 0.1% BMBM fortification level (Table 7), achieving 100% sensitivity (100% accurate identification of true-positive samples).

Comparison of MRTA and simplex real-time assay. In order to compare the new assay to the current validated simplex real-time assay, both assays were used to identify 10 BMBM, 10 OMBM, and 10 CMM samples, with each meal used to fortify control dairy feed at 0.1% inclusion rate. The MRTA assay identified all 30 samples as positive for ruminant DNA. The simplex real-time assay correctly identified 10 of 10 BMBM-fortified feed samples using the

TABLE 6. *Second and third analyst trials*[a]

Analyst/sample	Primer/probe set[b]	
	Ruminant	IAC
Second		
Control	0/15 (0.0)	15/15 (100)
BMBM	15/15 (100)	0/15 (0.0)
OMBM	15/15 (100)	0/15 (0.0)
CMM	15/15 (100)	0/15 (0.0)
Third		
Control	0/15 (0.0)	15/15 (100)
BMBM	15/15 (100)	0/15 (0.0)
OMBM	15/15 (100)	0/15 (0.0)
CMM	15/15 (100)	0/15 (0.0)
Success rate, second analyst		
True-negative rate	100%	100%
True-positive rate	100%	100%
Success rate, third analyst		
True-negative rate	100%	100%
True-positive rate	100%	100%

[a] IAC, internal amplification control; BMBM, bovine meat and bone meal–fortified feed; PMBM, porcine meat and bone meal–fortified feed; OMBM, ovine meat and bone meal–fortified feed; CMM, caprine meat meal–fortified feed. Dairy feed was fortified with BMBM, OMBM, and CMM at 0.1% (wt/wt). The fortified feed was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

[b] Results for positive feed samples (BMBM, OMBM, and CMM) are expressed as number of positive results/number of samples and accuracy (percentage of positive results in parentheses) for samples. Results for negative controls (control and PMBM containing feed) indicate the false-positive rate.

bovine primer set, 10 of 10 OMBM-fortified feed samples using the sheep primer set, and 10 of 10 CMM-fortified feed samples using the goat primer set (Table 8).

Single laboratory peer verification. Peer verification of the method was conducted at an independent laboratory. The analyst was trained and then given practice samples prior to beginning the actual trial. The results of the trial demonstrated that another laboratory could successfully perform this method. The verifying laboratory correctly identified 30 of 30 feed samples containing BMBM and demonstrated no false positives with the true-negative (control feed) samples (Table 9).

TABLE 7. *Results of multiplex with feed*[a]

Feed (0.1%)	Primer/probe set[b]	
	Ruminant	IAC
EU BMBM	30/30 (100)	0/30 (0.0)

[a] IAC, internal amplification control; EU, European Union; BMBM, bovine meat and bone meal–fortified feed. Dairy feed was fortified with BMBM rendered to EU specifications and added to control dairy feed at a 0.1% (wt/wt) rate of inclusion. The DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

[b] Values are number of positive samples/number of samples tested (percentage of positive samples in parentheses).

TABLE 8. *Comparison of the multiplex real-time assay to the simplex real-time assay*[a]

Sample (0.1%)	MRTA	SPRTA
BMBM	10/10	10/10
OMBM	10/10	10/10
CMM	10/10	10/10

[a] Values are the number of positive samples/total number of samples tested. MRTA, multiplex real-time PCR assay; SPRTA, simplex real-time PCR assay; BMBM, bovine meat and bone meal–fortified feed; OMBM, ovine meat and bone meal–fortified feed; CMM, caprine meat meal–fortified feed. Results of comparison between the MRTA and the current validated PCR assay used by the FDA, SPRTA, which used species-specific primers. The DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

DISCUSSION

To prevent the dissemination of BSE in the United States, the U.S. Department of Agriculture requires a valid permit to import any processed animal protein, while FDA regulations prohibit feeding specific mammalian-derived proteins to ruminants *(1)*. Some notable exceptions include pure porcine, pure equine, blood, milk, and "plate waste." The only assay capable of simultaneously detecting all possible processed animal proteins is microscopic examination. Microscopy is used to distinguish between processed animal proteins derived from avian, aquatic, or mammalian origin but cannot distinguish between species such as poultry (exempt) and pigeon (filth), or porcine (exempt) and bovine (prohibited). PCR has the discriminatory power to determine the species identity of the animal materials present in feed.

This study presents an evaluation of a MRTA capable of detecting bovine-, ovine-, and caprine-rendered DNA in feeds. The ChargeSwitch gDNA Rendered Meat Kit, optimized for use in animal meal and animal feed, was used in this study. It was found to be relatively trouble free

TABLE 9. *Peer-verification results*[a]

Feed	Primer/probe set[b]	
	Ruminant	IAC
Control	1/30 (3.33)	29/30
BMBM	30/30 (100)	0/30

[a] IAC, internal amplification control; BMBM, bovine meat and bone meal–fortified feed. Dairy feed was fortified with rendered BMBM at 0.1% (wt/wt). The DNA was extracted using the ChargeSwitch gDNA Rendered Meat Purification Kit and amplified with the MRTA using a Smart Cycler II.

[b] Values are number of positive samples/number of samples tested (percentage of positive samples in parentheses).

compared with previous extraction methods *(5–7)* and is the FDA's current validated regulatory method for DNA extraction from feed and feed ingredients *(11)*. The evaluation of the MRTA included an assessment of the specificity, sensitivity, selectivity, and ruggedness of the multiplex assay. Additional trials of the multiplex assay were successfully conducted, in a blinded manner, by in-house second and third analysts, as well as by an external laboratory for peer verification. The MRTA successfully passed the in-house evaluation as well as the external peer verification.

The MRTA was evaluated against the current validated simplex real-time PCR assay in a bridging study to determine assay comparability. The MRTA demonstrated the ability to reliably detect the three prohibited ruminant processed proteins in dairy feed enriched with 0.1% of each of the prohibited species' respective MBM. This shows that the MRTA meets or exceeds the level of previous PCR-based assays validated by the FDA *(6, 7, 11)*. The data illustrate that the multiplex assay accurately detected only the three target species (without cross-reacting with any of the other tested species) and was capable of detecting as little as 0.1% animal protein in complete feed (the level of sensitivity of previous PCR-based assays validated by the FDA *(6, 7, 11)*). The assay protocol demonstrated ruggedness both in the selection of samples and in the preparation of PCR reagents. Second and third analyst trials determined that the standard operating procedure for this method was adequate, with no changes needing to be incorporated, and demonstrated the reproducibility of results between analysts. The results demonstrated that the assay could successfully detect DNA found in authentic EU animal meals in complete feed. The MRTA meets all of the criteria developed by the FDA. Because this multiplex PCR-based assay can detect three relevant ruminant species of concern simultaneously, it could be used as a screening tool or as a confirmatory assay. The MRTA described could allow for the replacement of the current simplex real-time PCR assay to improve the speed and accuracy of feed and meal testing. The MRTA is able to determine the presence of the prohibited processed animal proteins in meal and feed that are derived from meals prepared in North America or in the EU. It is capable of providing additional assurances for feed safety above and beyond what is currently available with existing assays and holds possibilities for enhancing the enforcement of existing regulations.

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