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Research Note

Detection of Prohibited Animal Products in Livestock Feeds by Single-Strand Conformation Polymorphism Analysis†‡

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ABSTRACT

Single-strand conformation polymorphism (SSCP) analysis of amplicons produced from a mitochondrial DNA region between the *tRNA*^{Lys} and *ATPase8* genes was applied for the detection of animal product within livestock feeds. Identification of prohibited animal (cattle, elk, sheep, deer, and goat) and nonprohibited animal (pig and horse) products from North America was possible based on the differential display of the single-stranded DNA fragments for the different animal species on SSCP gels. This method allowed specific detection and identification of mixed genomic DNA from different animal species. Trace amounts of cattle-derived materials were also detected in pig meat and bone meal and in grain-based feeds fortified with 10, 5, 1, or 0% porcine meat and bone meal. This study demonstrates the applicability of SSCP analyses to successfully identify the origin of animal species derived materials potentially present in animal feeds.

In response to the emergence of bovine spongiform encephalopathy, many nations have implemented comprehensive regulations prohibiting the feeding of many mammalian materials, including meat and bone meal (MBM), to ruminants. In North America, materials of equine and porcine origin are permitted in ruminant feeds. It is very important to know the animal species origin of mammalian material detected in feeds, other than the two exempt mammalian species. Currently, microscopic examination of animal feed is widely regarded as the method of choice for the determination of prohibited materials *(7)*. This method is very effective in identifying the presence of minute amounts of meat and bone materials. Microscopy, however, does not allow for determination of the species origin of meat or bone materials. Also, microscopy is a time-consuming, labor-intensive method. In order to meet the requirement for more sensitive and specific methods to rapidly identify the species origin of the derived materials, several molecular assays have been developed using PCR-based restriction fragment length polymorphism *(2, 12)* and single-strand conformation polymorphism (SSCP) analyses *(14, 16),* real-time PCR *(10),* and a cloth-based hybridization array system (CHAS) *(1, 11)*. Some of these methods are based solely on the detection of bovine material *(10, 16),* whereas others are more sensitive, with the potential of detecting multiple prohibited species *(1, 2, 11, 12, 14)* and some permitted species *(1, 2, 11, 12)*. The CHAS procedure appeared to provide the more informative results for risk assessment purposes. The sensitivity of detection was high, and the identification of DNA from five prohibited animal species materials (cattle, sheep, goat, elk, and deer) *(1)* and two permitted animal species (pig and horse) was achieved when simulated meat meals were prepared under typical Canadian and U.S. rendering conditions *(11)*. As a consequence, the CHAS method seems to have the potential to be a useful adjunct to microscopic examination for the identification of prohibited materials in animal feeds, along with other screening methods *(11)*. However, the development of complementary techniques to further analyze feeds, particularly in the presence of more complex feed samples, is essential in order to gain more information on the status of samples for risk assessment purposes and prevent adulteration with prohibited materials.

SSCP has only been used on a limited scale to verify specific amplification of the *ATPase8* gene with an *ATPase6* fragment from the mitochondrial (mt) DNA from reference feeds and bovine genomic DNA (gDNA) *(16)* or to determine the species origin of the *tRNA*^{Lys}*-ATPase8* mtDNA amplified from the gDNA of different species within the Artiodactyla *(14)*. The *tRNA*^{Lys}*-ATPase8* mtDNA has become an established target for discrimination of various ruminant and nonruminant species *(1, 12, 16)* based on previously determined sequence data *(4, 14, 15)*. Given that SSCP is a simple and cost-effective method *(5),* we investigated in the present study the potential of SSCP for

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the detection and identification of prohibited ruminant materials in animal feeds. We characterized the SSCP profiles of the *tRNA*^{Lys} and *ATPase8* mtDNA gene fragment from gDNA of five prohibited animal species (cattle, elk, deer, goat, and sheep) and two permitted species (pig and horse), by use of ''universal primers'' *(14)*. The ability to detect trace amounts of cattle-derived materials by using SSCP was evaluated with rendered meat meal blends and grain-based feeds fortified with pig MBM.

MATERIALS AND METHODS

The conditions for sample preparation, DNA extraction, and PCR amplification were optimized in previous studies *(1, 11,* unpublished data*)*.

Preparation of gDNA from meat samples. The origin and the preparation of meat samples from cattle (*Bos taurus*), sheep (*Ovis aries*), elk (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*), goat (*Capra hircus*), pig (*Sus scrofa*), and horse (*Equus caballus*) were the same as previously described *(1, 11)*. In brief, meat meals from each animal species were prepared individually by cutting the meats into small cubes, followed by processing at 121°C in an autoclave (1 atm [101.29 kPa]) for 20 min in order to simulate rendering conditions. The cooked meats were then frozen at −80°C and freeze-dried for ease of subsequent grinding. These samples were ground with a mortar and pestle to achieve a fine particle size resembling the consistency of commercial meat meals. gDNA was extracted from approximately 100 mg of each of the simulated meat meals by using the ChargeSwitch gDNA rendered meat purification kit (Invitrogen, Carlsbad, CA) with a MagnaRack Magnetic Rack (Invitrogen) for the manual purification procedure. The extraction was performed as described in the protocol supplied with the kit, using elution buffer prewarmed to 60°C to increase the DNA yield. The gDNA was quantified using a SYBR Green I microplate fluorescence assay as described previously *(9)*. The gDNA was then diluted to 5 ng/μl.

Preparation of rendered meat meal blends of pig and cattle MBM. Samples of cattle MBM and pig MBM were collected from Canadian rendering plants by Canadian Food Inspection Agency inspectors. The samples were screened for purity using the PCR and CHAS method as previously described *(1, 11)* prior to preparing the blends to confirm the authenticity of the samples (data not shown). Blends of pig MBM containing defined levels of cattle MBM (0.1, 0.05, 0.025, 0.013, 0.006, 0.003, and 0.0015% [wt/wt]) were prepared as follows: a 10.0% (wt/wt) blend was prepared by adding 1.0 g of cattle MBM to 9 g of pig MBM in a 50-ml screw-cap tube and then mixed in a Turbula Mixer (model T2F, Glen Mills, Inc., Clifton, NJ) for 30 min. Blends containing lower levels of cattle MBM were prepared by successive serial dilutions with pig MBM and mixing as described above. The 10 g of MBM used to prepare the samples was sufficient, as these blends were not used for the creation of other blends.

Preparation of grain-based feeds fortified with pig MBM containing cattle MBM. A blank feed matrix (BFM) (grain-based feed devoid of animal by-products) into which simulated meat meals were blended was comprised of a soybean meal and Beef Chop (consisting of corn, barley, and molasses) purchased from local retailers and blended into a fine powdery consistency as previously described *(1)*. Blends of BFM fortified with defined levels of pig MBM (10.0, 5.0, or 1.0% [wt/wt]) were prepared as follows: to make a 10.0% (wt/wt) blend, 3.5 g of pig MBM was mixed as above with 31.5 g of BFM in a 250-ml screw-cap container. This total amount of 35 g was chosen to have enough material for the preparation of other blends prepared as follows: to make the 5.0% (wt/wt) blend, 1.6 g of pig MBM was added to 30.4 g of BFM in a 250-ml screw-cap container, followed by mixing as described above. To make the 1.0% (wt/wt) blend, 3.0 g of the 10.0% pig MBM in BFM (prepared as described above) was added to 27.0 g of BFM in a 250-ml screw-cap container, followed by mixing as above. To determine the limit of detection for cattle MBM in BFM containing different levels of pig material, blends of BFM fortified with 10.0, 5.0, or 1.0% (wt/wt) pig MBM containing defined levels of cattle MBM (0.1, 0.01, 0.001, and 0.0001% [wt/wt]) were prepared as follows: a 10.0% (wt/wt) cattle MBM in the presence of 10% (wt/wt) pig MBM blend was made by adding 0.3 g of pig MBM to 2.4 g of BFM in a 50-ml screw-cap tube, followed by the addition of 0.3 g of bovine MBM, and the blend was mixed as described above. A 10% (wt/wt) cattle MBM in 5.0% (wt/wt) pig MBM blend was made by adding 0.15 g of pig MBM to 2.55 g of BFM in a 50-ml screw-cap tube, followed by the addition of 0.3 g of bovine MBM, and the blend was mixed as above. Successive serial dilutions with the appropriate background matrix containing 10.0, 5.0, or 1.0% (wt/wt) pig MBM in BFM and mixing as described above were made to prepare blends containing lower levels of cattle MBM in 10.0, 5.0, or 1.0% (wt/wt) pig MBM.

Preparation of nonfortified grain-based feeds containing cattle MBM. To determine the limit of detection for cattle MBM in BFM devoid of other animal materials, blends of blank feed matrix (nonfortified) containing defined levels of cattle MBM (0.1, 0.01, 0.001, and 0.0001% [wt/wt]) were prepared as follows: to make the 10.0% (wt/wt) cattle MBM in BFM blend, 0.7 g of cattle MBM was mixed as described above with 6.3 g of BFM in a 50-ml screw-cap tube. Blends containing lower levels of cattle MBM were prepared by successive serial dilutions with BFM and mixing as described above.

Extraction of DNA from the simulated feed blends. DNA was extracted from approximately 250 mg of each of the bovine-in-porcine MBM blends, or 500 mg (2 × 250 mg, pooled following the lysis step) of the MBM in feed blends *(1)* by using the ChargeSwitch gDNA Rendered meat purification kit and the MagnaRack Magnetic Rack for the manual purification procedure. The extraction was performed as described in the protocol supplied with the kit, using elution buffer prewarmed to 60°C to increase the DNA yield. The DNA concentration was not quantified.

PCR. All primer sequences used in this study were synthesized by a commercial contractor (Alpha DNA, Montreal, Quebec, Canada). A 270-bp region of the mt *tRNA*^{Lys} and *ATPase8* genes *(12, 14)* was amplified by PCR as described previously *(1),* but without the use of the DIG-11-dUTP. PCRs were carried out in volumes of 100 μl containing 2.5 U of HotStar *Taq* polymerase (Qiagen), 1× PCR buffer, 4.5 mM $MgCl_2$, 50 mM Tris [pH 8.3], 0.02 μg of bovine serum albumin per μl, 200 μM (each) dinucleoside triphosphate, 0.4 μM (each) universal primer (L8049, 5′-ACT TTG AAA AAT GAT CTG CAT CAA-3′ and H8273, 5′-TCG TTC ATT TTG TTT CTC AAG GGG T-3′), and 10 μl of DNA. PCRs were carried out in a GeneAmp PCR system 9700 thermocycler as follows: 94°C for 15 min, followed by 31 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and then 72°C for 10 min.

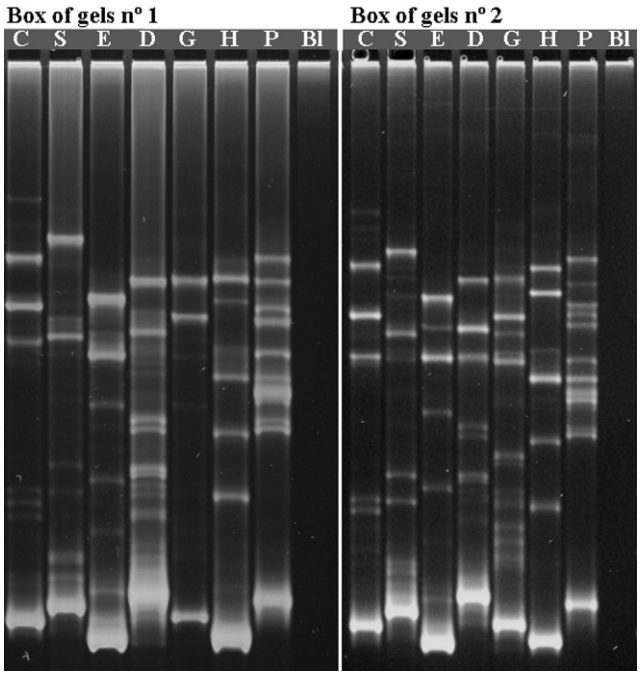

FIGURE 1. *SSCP profiles of L8049/H8273 mtDNA amplicons from gDNA from different animal species. C, cattle; S, sheep; E, elk; D, deer; G, goat; H, horse; P, pig; Bl, extraction blank.*

SSCP analyses. SSCP was performed for each sample as described previously *(6)*. In brief, denatured amplicons were mixed with tracking dye (Promega, Madison, WI) and loaded into the wells of precast GMA Wide Mini S-50 gels (Elchrom Scientific, Cham, Switzerland). Gels were run at 74 V for 17 h in a horizontal SEA2000 electrophoretic apparatus connected to a circulating water bath (Elchrom Scientific), which maintained a constant water temperature of 8°C. SSCP gels were then stained with SYBR Gold (Invitrogen Life Technologies, Burlington, Canada) for 40 min and destained in H_2O for 20 min prior to examination with UV light. Photographic records of each SSCP gel were made using a gel documentation system (Alpha Innotech Corporation). Amplicons from gDNA of each animal species were used as controls in SSCP analyses of PCR products from mixed gDNA and from those generated from gDNA of the simulated feeds.

RESULTS

The *tRNA*^{Lys}*-ATPase8* mtDNA gene region was amplified by PCR from gDNA of each sample of meat originating from different species (cattle, sheep, deer, elk, goat, pig, and horse), from gDNA mixed combining two animal species origins, and from gDNA of simulated rendered meat meal blends containing different percentages of bovine materials in porcine MBM, or in a grain-based feed fortified with 10, 5, or 1% porcine MBM. For the nonfortified grain-based feeds, amplification was successful only when bovine material was present at 0.1 and 0.01%. The amplicons were of the expected molecular size as detected on agarose–Tris-borate-EDTA gels (data not shown). For the simulated rendered meat blends, from which the gDNA has not been quantified, a 10-μl volume of gDNA in a PCR was sufficient to produce amplicon suitable for SSCP analysis.

The SSCP analysis of the amplicons generated using primers L8049/H8273 displayed considerable variations in the banding patterns among the different animal species (Fig. 1). The number of bands displayed for the amplicons generated from each animal species varied from 2 to 13. Each species had a unique SSCP profile. The banding patterns for the amplicons generated from each of the species were reproducible upon reamplification, as well as

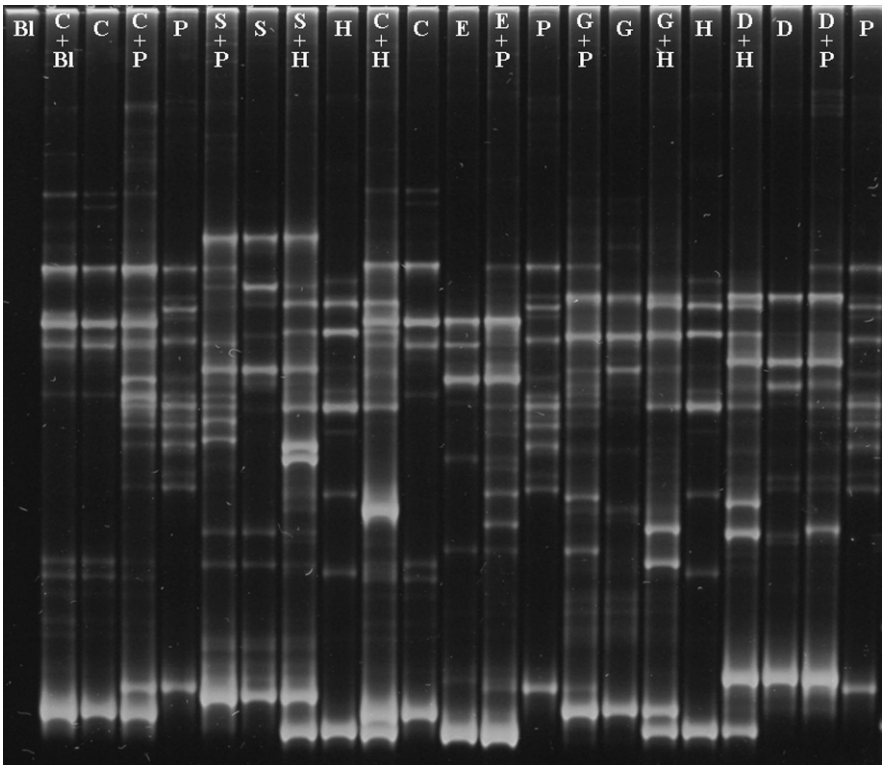

FIGURE 2. *SSCP profiles of L8049/H8273 mtDNA amplicons from equimolar mixed gDNA (12.5 ng each) from different animal species. Bl, extraction blank; C, cattle; P, pig; S, sheep; H, horse; E, elk; G, goat; D, deer; C+Bl, cattle and extraction blank; C+P, cattle and pig; S+P, sheep and pig; S+H, sheep and horse; C+H, cattle and horse; E+P, elk and pig; G+P, goat and pig; G+H, goat and horse; D+H, deer and horse; D+P, deer and pig.*

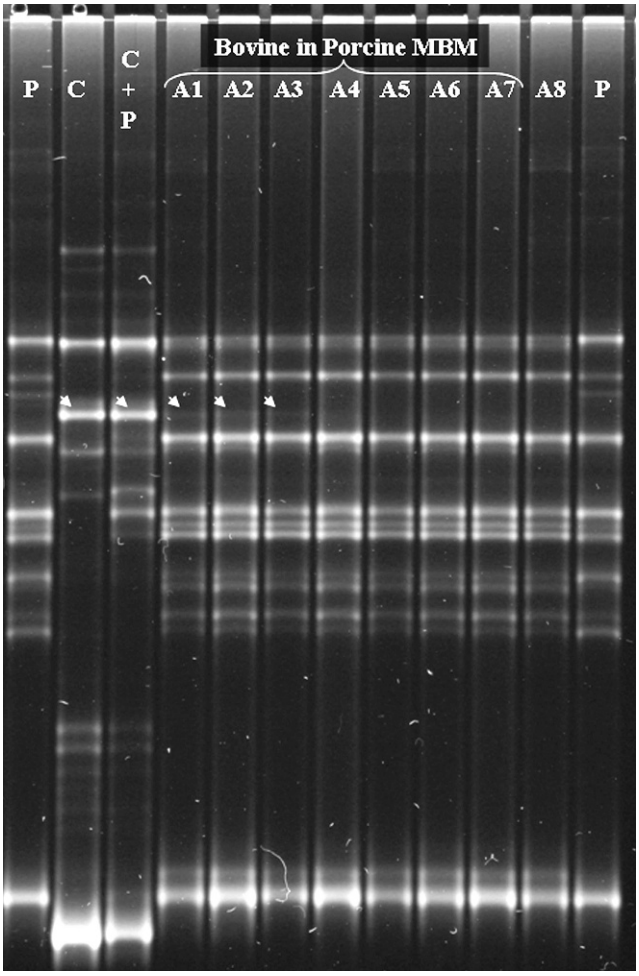

FIGURE 3. *SSCP profiles of L8049/H8273 mtDNA amplicons from gDNA extracted from simulated porcine MBM containing different percentages (wt/wt) of cattle-derived materials: 0.1% (A1), 0.05% (A2), 0.025% (A3), 0.013% (A4), 0.006% (A5), 0.003% (A6), 0.0015% (A7), and 0% (A8). Controls consisted of L8049/H8273 mtDNA amplicons from gDNA from cattle (C), pig (P), and mixed gDNA (12.5 ng each) of cattle and pig (C+P). Arrows show single-strand bands characteristic to the cattle SSCP profile.*

within a gel (Fig. 2, profiles C, P, and H), and were consistently detected on a total of 32 gels (data not shown). However, a slight variation in the resolution of the SSCP profiles was noticed between two different sets of the commercially available SSCP gels. This consisted of the display of additional bands for the goat, the elk, and the deer meat samples and a small variation in the position of the bands for the cattle meat sample (Fig. 1). The banding patterns obtained with amplicons from mixed gDNA of different combinations of two animal species were more complex with a superimposition of the major single-strand bands of the SSCP profiles originating from the two animal species, as well as the formation of additional bands on the lower portion of the gels for some combinations (S+H, C+H, and E+P; Fig. 2). SSCP profiles obtained with combinations of gDNA from two different animal species were also consistent over a total of 21 gels (data not shown). For the simulated rendered meat meal blends, the SSCP banding pattern characteristic of the pig amplicon was detected in the porcine MBM (A1 to A8; Fig. 3) and in all fortified feed samples (B1 to B5, C1 to C5, and D1 to D5; Fig. 4). Additional bands corresponding to the SSCP pattern characteristic of the bovine amplicon were clearly detectable at the highest percentages of bovine material present: from 0.1 to 0.025% in the porcine MBM (A1 to A3; Fig. 3) and from 0.1 to 0.01% in the fortified and nonfortified feed samples (B1, C1 and C2, D1 and D2, and E1 and E2; Fig. 4). However, faint bands were also visible in two of three gels, for lower percentages (0.01 to 0.0001%) of bovine material in the fortified feed blends (B2 and B3, C2, and D3 and D4; Fig. 4).

DISCUSSION

Although previous studies have used SSCP to detect a bovine-specific mtDNA (*tRNA*^{Lys}*-ATPase8* mtDNA) sequence *(16)* or to determine the species origin of amplicons by using universal primers *(14),* the SSCP method has not been used to detect and identify multiple prohibited and nonprohibited species in feed samples. This would provide more detailed information on species origin for the purposes of investigation, traceback, and risk assessment. In our study, we used the universal PCR method *(12, 14),* also previously employed in the CHAS method recently developed *(1, 11),* followed by an SSCP assay using commercial gels to analyze the amplicons. Our results showed that all the animal species tested could be readily distinguished from one another based on their SSCP profiles (Fig. 1). The variation in the resolution of SSCP banding patterns between two different batches of commercially manufactured gels did not affect the analysis of the amplicons. However, this variation showed the importance of including control samples (i.e., amplicons from each animal species origin) on each gel during SSCP analyses of feed samples that could contain unknown animal-derived materials. Thus, identification of the animal species origin in samples would be based on the detection of the single-stand bands specific to each animal species used as controls.

The SSCP banding patterns (i.e., profiles) of amplicons from mixed gDNA combining two species origins consisted of the combination of the banding patterns of both animal origins (Fig. 2). However, the presence of additional bands was noticed on the lower part of the gels, probably due to the formation of additional heteroduplexes between single strands. The presence of these heteroduplexes was consistent over a total of 10 gels (data not shown). However, these heteroduplexes were not detected when one of the animal species origins is in excess of another species (data not shown), or when both animal species are present in trace amounts (Fig. 3, lanes A1 to A7, and Fig. 4, lanes D1 to E2). Therefore, the identification of the two animal species origins present in more complex feeds should be based on the analysis of the single-strand bands (upper bands) of the SSCP profiles. These results showed that SSCP has the potential to detect and identify the animal species origin in more complex feeds. In order to further demonstrate this

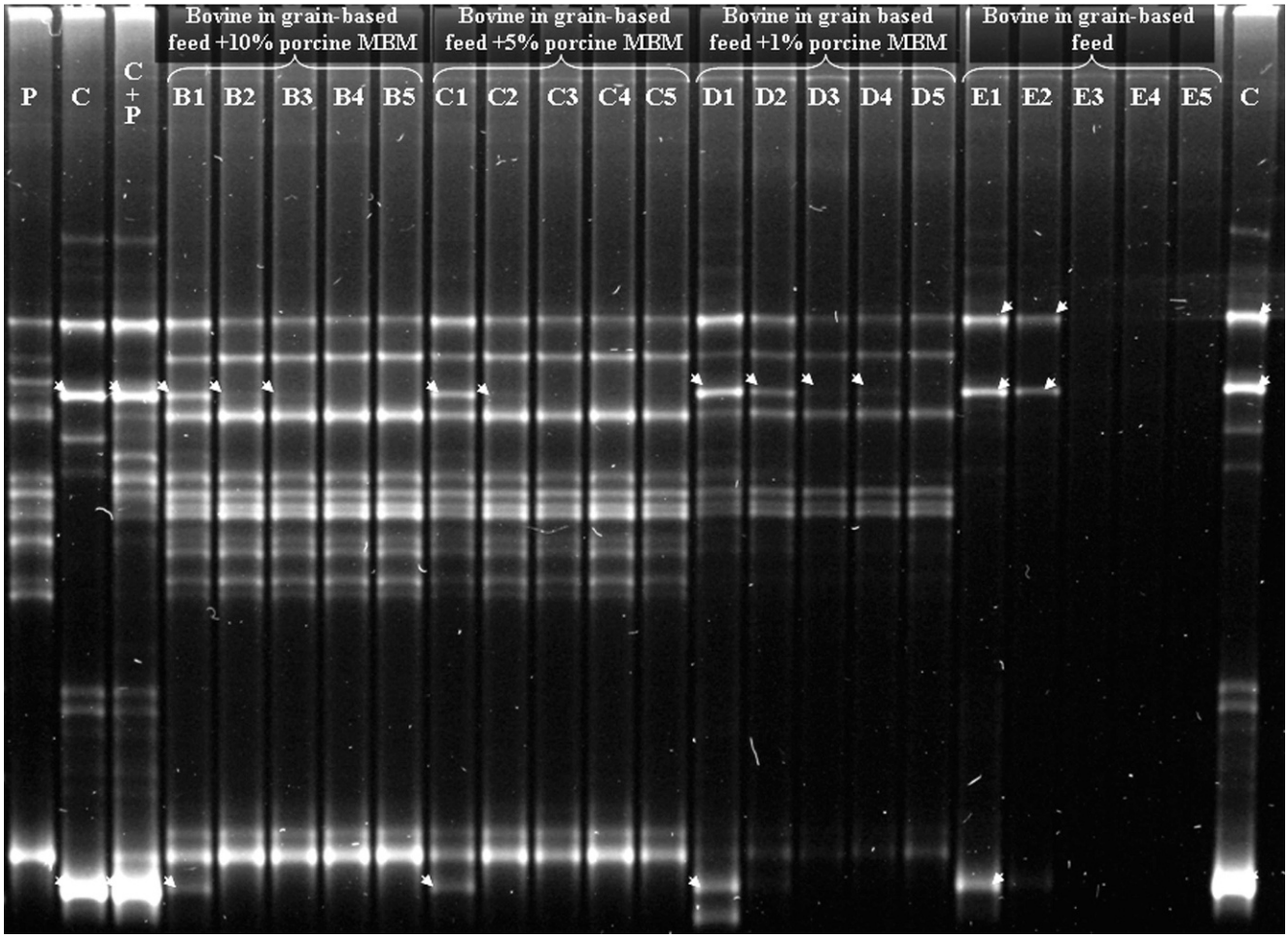

FIGURE 4. *SSCP profiles of L8049/H8273 mtDNA amplicons from gDNA extracted from simulated grain-based feed fortified with 10, 5, 1, or 0% porcine MBM, containing different percentages (wt/wt) of cattle-derived materials: 0.1% for B1, C1, D1, and E1; 0.01% for B2, C2, D2, and E2; 0.001% for B3, C3, D3, and E3; 0.0001% for B4, C4, D4, and E4; and 0% for B5, C5, D5, and E5; controls consisted of L8049/H8273 mtDNA amplicons from gDNA from cattle (C), pig (P), and mixed gDNA (12.5 ng each) of cattle and pig (C+P). Arrows show single-strand bands characteristic to the cattle SSCP profile.*

potential, gDNAs from different simulated feed samples were extracted and SSCP analyses were conducted to determine whether this technique could detect trace amounts of a prohibited animal species origin in presence of an excess of a permitted species.

SSCP analyses of simulated rendered blends showed the detection of both characteristic profiles of bovine and pig amplicons (Figs. 3 and 4). However, the detection of the prohibited species was dependent on the percentages of the bovine and pig MBM present in the feeds. Considering the faint bands, the detection limit was achieved on one gel, at 0.0001% of bovine materials in 1% pig MBM (Fig. 4), and at 0.025% of bovine materials in porcine MBM (Fig. 3). Based on the results from several gels, the experiment showed that when the concentration of porcine MBM is lower, the bovine MBM contaminant can be detected at lower concentrations. Similar detection limits were reported with the CHAS system, with which the ability to detect one species was also negatively impacted in the presence of an excess of another species targeted by the CHAS *(1, 11)*. Lower sensitivities of the detection of ruminant materials in feed were reported with the use of real-time PCR. The detection limits were 0.1% *(13)*, 0.125% *(3)*, and 0.01% *(8)* of bovine MBM when present in fishmeal.

The results of the present study emphasize the proof of concept of this assay to detect and identify trace amounts of materials derived from prohibited ruminant species in rendered feed samples. Further validation with more complex experimental MBM samples is required. However, this study showed the potential of this assay to be used as a tool, along with other methods, such as the CHAS system and real-time PCR, to analyze feeds for which suspect samples have been identified by microscopy, and thereby aid in control of the spread of transmissible spongiform encephalopathies.

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