

## Detecting meat-and-bone meal in ruminant's feeds by species-specific PCR\*

**Y.H. Cheng<sup>1</sup>, C.M. Wen<sup>3</sup>, S.T. Ding<sup>2</sup>, C.C. Kao<sup>1</sup> and T.Y. Kuo<sup>4</sup>**

<sup>1</sup>*Department of Animal Science, National I-Lan University  
#1, Sheng-Lung Rd, I-Lan 260, Taiwan*

<sup>2</sup>*Department of Animal Science, National Taiwan University  
Taipei, Taiwan*

<sup>3</sup>*Department of Life Science, National Kao-Hsiung University  
Kao-Hsiung, Taiwan*

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### ABSTRACT

Bovine spongiform encephalopathy, first identified in the UK in 1986, may have arisen from feeding scrapie infected meat-and-bone meal (MBM) to cattle that was produced under sub-optimal conditions. For public health and disease prevention reasons, banning the application of MBM in ruminant's feeds was necessary. This study utilized a polymerase chain reaction (PCR) assay to identify bovine, porcine, ovine, and chicken MBM in animal diets. Bone fragments from feed samples were separated by chloroform separation. A nucleospin column was used for DNA extraction. Four pairs of primers that targeted at highly conserved regions of mitochondrial DNA (mtDNA) were employed in a PCR procedure to detect bovine, porcine, ovine and chicken DNA sequences. These gene fragments at the targeting region for the four species were 271, 225, 212 and 266 bps in size, respectively.

In order to confirm the specific amplification of expected products from MBM for each species, the PCR products were digested with restriction enzymes HphI, MnlI, SspI and HindIII, and different length polymorphisms were observed. In the sensitivity assays, concentration as low as 1% of MBM in animal feeds could be detected by the PCR procedure. Based on our prevalence survey for commercial MBM in Taiwan, it predominantly consisted of bovine or porcine origins. This molecular approach provides a quick, highly reproducible and sensitive method to detect the MBM in feeds.

KEY WORDS: PCR, meat-and-bone meal, feeds, detection

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<sup>4</sup> Corresponding author: e-mail: tykuo@niu.edu.tw

## INTRODUCTION

Bovine spongiform encephalopathy (BSE) also known as mad cow disease, is a fatal progressive and neuro-degenerative disease. The first BSE breakout in UK was in 1986. It was due to feeding cattle with rendered protein supplement derived from scrapie-infected meat-and-bone meal from sheep (Wells et al., 1987). For preventing spreading of BSE from cattle to other domestic animals, a ban on enriching cattle feeds with ruminant-derived proteins started in 1988 in UK. Similar regulations that banned the use of mammalian-derived proteins for ruminant feed was implemented in European countries and the United States of America, to prevent the spreading of the prion from animal products to humans (Baron et al., 1999). In 1996, a new-variant Creutzfeldt-Jakob disease (nvCJD) and kuru disease was identified and it was proposed that these diseases can transmit to humans (Will, 1999). These observations indicate new threatening originated from ruminant-derived proteins to humans.

A strict guideline was established for animal offal and wastes rendering process, which included hyperbaric treatment at 133°C/3 bar for 20 min, after initial processing of the material at 120°C and removal of the tallow (Decision 96/449/ European Commission, 1996).

So far, meat-and-bone meal (MBM) identification from feeds was mainly relying on microscopic examination. However, this approach not only needs well-trained personnel but also is time-consuming. During the last few years, study have demonstrated that the utilization of PCR for the detection of a very small number of DNA molecules was possible (Taberlet et al., 1996). Such approach can amplify the gene sequence of interest by using PCR. The PCR technique is a time-sparing and sensitive method to identify gene sequences of species-specific in raw meat (Gouli et al., 1999; Matsunga et al., 1999), processed fish meat (Mackie et al., 1999), and bovine-derived MBM (Tartaglia et al., 1998; Lahiff et al., 2001).

The PCR method used in this study was focusing on ATPase subunit 6 and subunit 8 of highly conserved regions from mtDNA (Colgan et al., 2001). Wang et al. (2000) reported that utilizing chelex-100 for DNA extraction along with specific primers for PCR, can be applied to identify bovine mtDNA within 2 h. Therefore, PCR is a quick, easy and highly sensitive method. The aims of this study were to identify original species of MBM supplemented in ruminant feeds by PCR, duplex PCR to detect bovine and porcine MBM mixture. The prevalence of MBM supplemented in commercial ruminant feeds in Taiwan was also determined.

MATERIAL AND METHODS

*Isolation of mtDNA*

Bone fragments of feed samples were separated by suspended feeds in chloroform solution (specific gravity 1.48-1.50). Down-layered bone fragments were dried in heated oven (55°C, 15 min) and then used for mtDNA extraction. Chloroform specific gravity approach was utilized to separate inorganic matter from organic in order to enhance PCR results by avoiding the interference from polymerase inhibitors in feeds.

Feed samples (0.2 g) were mixed with 1ml of a 20% Chelex-100 (Bio-Rad Laboratories, Hercules, CA) in 1% Triton X-100. The 20% Chelex-suspension was mixed well with each sample. The samples were placed in a water bath at 90-95°C for 15 min, then placed into ice water for 1 min and centrifuged at 10,000 × g for 5 min. Another DNA extraction method utilizing a nucleospin column (Macherey-Nagel, Duren, Germany) was also employed, following the procedure in the manufacturer’s menu.

*PCR amplification*

Two microliters of the supernatant (template DNA) was added to 23 µl of PCR mixture containing 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM MgCl<sub>2</sub>, 0.05% bovine serum albumin (BSA, No. A-4378, SIGMA Chemical, St Louis, MO), 0.2 mM of dNTP, 0.25 µM of each primer (Table 1), 2 U of Taq DNA polymerase. The PCR was performed in a thermocycler (GeneAmp PCR System 2400, Applied

TABLE 1

Oligo-nucleotide primer sequences for different species

Primer sequence	Amplicon length (bp)	Source
<i>Bovine</i>		
5'GCCATATACTCTCCTTGGTGACA 3'	271 bp	Tartaglia et al. (1998)
5'GTAGGCTTGGGAATAGTACGA 3'		Tartaglia et al. (1998)
<i>Porcine</i>		
5'GCCTAAATCTCCCCTCAATGGTA 3'	212 bp	Lahiff et al. (2001)
5'ATGAAAGAGGCAAATAGATTTTCG 3'		Lahiff et al. (2001)
<i>Ovine</i>		
5'TTAAAGACTGAGAGCATGATA 3'	225 bp	Lahiff et al. (2001)
5' ATGAAAGAGGCAAATAGATTTTCG 3'		Lahiff et al. (2001)
<i>Chicken</i>		
5'GGGACACCCTCCCCCTTAATGACA3'	266 bp	Tartaglia et al. (1998)
5'GGAGGGCTGGAAGAAGGAGTG 3'		Lahiff et al. (2001)

Biosystems, Singapore) using the following conditions: an initial denaturation step at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min for 30 cycles followed by a final extension time at 72°C for 5 min. PCR and extraction negative controls were included for each amplification experiment in order to detect false positives due to contamination.

PCR products were examined by electrophoresis through a 2% agarose (Agarose LE, Promega) gel in 1×TBE buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001M EDTA, pH 8.0) and stained by ethidium bromide. A 100 bp DNA ladder marker was used as size reference.

### *Experiment treatment*

*Inclusion test of oligonucleotide primers.* DNA was extracted from beef, mutton, pork and chicken meat as positive control throughout this study. Meat (0.2 g) was digested with 400 µl digestion buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA in 0.5% SDS, containing 20 mg/ml proteinase K) overnight at 37°C. Samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) solution and total DNA in the aqueous phase was precipitated with 1/10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes cold 100% ethanol at 4°C for 30 min. DNA pellets were obtained by centrifugation (13,000 × g, 10 min), washed twice each with 70 and 100% ethanol, air dried, then resuspended in 50 µl TE buffer and nucleic acid concentration was measured by spectrophotometry at 260 nm. Animal-derived supplement free feed were mixed with 5, 4, 3, 2 and 1% of MBM or poultry meal for the inclusion test.

*PCR-restriction fragment length polymorphism (RFLP) analysis.* For PCR-RFLP analysis, the procedure from Lahiff et al. (2001) was followed. Briefly, PCR products of bovine, porcine, ovine and chicken were digested with HphI, MnlI, SspI and Hind III restriction enzymes, respectively. The digested products were analysed in 3% Nusieve gel/1 × TBE buffer electrophoresis.

*Commercial ruminant feeds survey.* Commercial MBM or poultry meal samples were collected in Taiwan from Jan./2001 to Dec./2002, and each sample was extracted by two different extraction protocols. For prevalence survey, total of 277 samples were analysed, and the MBM contaminated percentage was calculated.

## RESULTS AND DISCUSSION

### *Inclusion test of oligonucleotide primers*

In the inclusion rate test, the detecting sensitivity as low as 1% of poultry meal was detected (Figure 1). Different species-specific primers (beef, pork and mut-

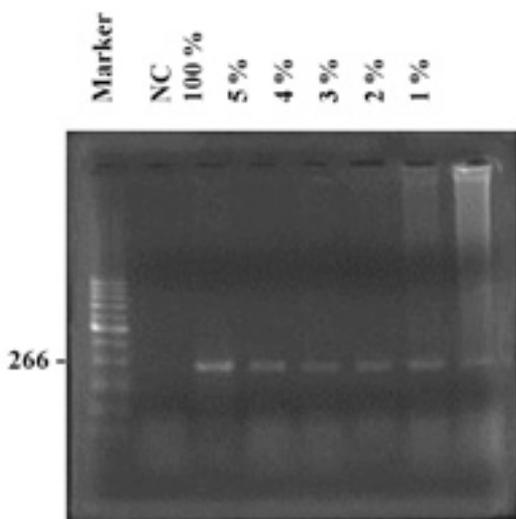


Figure 1. Detecting the poultry meal in feeds by PCR with chicken specific primer. Lane 1:100 bps ladder markers; Lane 2: negative control; Lane 3-8: 100, 5, 4, 3, 2 and 1% poultry meal was included in feeds, respectively

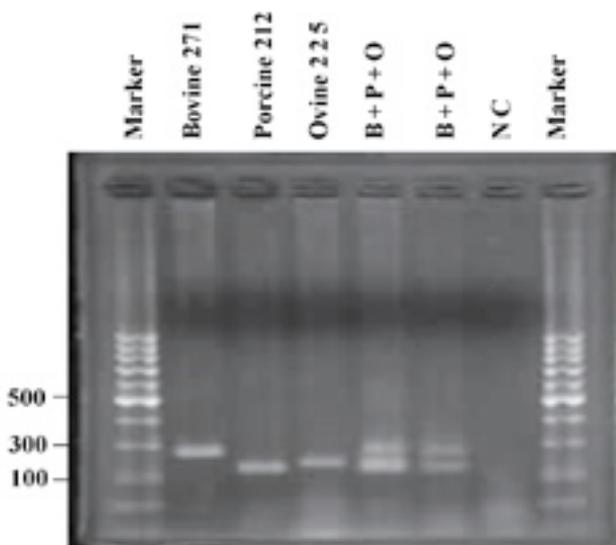


Figure 2. Different species-specific primers test for detecting beef, pork and mutton by PCR. Lane 1,8: 100 bps ladder markers ; Lane 2: beef; Lane 3: pork; Lane 4: mutton and Lane 5,6: beef+pork and mutton; Lane 7: negative control

ton) for PCR produced 271, 212 and 225 bps amplicons, respectively (Figure 2). The detection limitation for these three species were also 1%. The PCR products were cloned and sequenced. The sequences from the three species showed full match with DNA sequences in Genbank (Accession number J01394, AF039170, and AF039171). However, we have improved the detection sensitivity to 1% for poultry-specific procedure, which is much lower than what was reported by Lahiff et al. (2001).

Our investigation also revealed that the MBM available in market mostly composed of bovine and porcine mixture. Using duplex PCR and modified the annealing temperature could precisely detect the bovine and porcine mixed MBM, and detecting sensitivity was as low as 1% in feeds (Figure 3). Our results were the first to identify the bovine and porcine-derived MBM at the same time by duplex PCR (all reagents and procedures were the same as PCR amplification in material and methods, except the bovine and porcine specific primers were added). This method provides more precise detection of species-origin for MBM, and is more labour-saving than using each species-specific primers separately for the same samples.

#### *PCR-RFLP analysis*

The PCR products after digestion with HphI, MnlI, SspI, and HindIII restriction enzymes, were visualized in 3% Nusieve gel. The allelic polymorphism for bovine-derived, porcine-derived, ovine-derived, and chicken-derived MBM, were 271/250 + 21, 212/196 + 16, 225/143 + 83, and 266/187 + 79 bps, respectively (Figure 4). These results were similar to that of Lahiff et al. (2001), who established RFLP procedure to confirm the amplicon. However, the small fragments were difficult to visualize after restriction enzyme digestion, and high concentration of Nusieve gel were also hard to prepare. The polyacrylamide gel may be a better matrix for RFLP analysis.

#### *Commercial ruminant feeds survey*

Total of 277 ruminant feed samples were collected in Taiwan during year of 2001-2002. Twenty-four out of 277 samples were detected to be contaminated with bovine-derived products, 19 out of 277 samples contaminated with porcine products, and 5 out of 277 samples contaminated with both bovine and porcine products. The prevalence for bovine products was  $24/277 = 8.7\%$ , and for porcine products was  $19/277 = 6.9\%$  (Table 2). None of the 277 samples contained ovine-derived or chicken-derived products.

In the current study, we also compared chelex-100 method and the commercial kit (Nucleospin food column) for DNA extraction efficiency from MBM and feeds. We found that the Nucleospin kit was better for DNA extraction than the

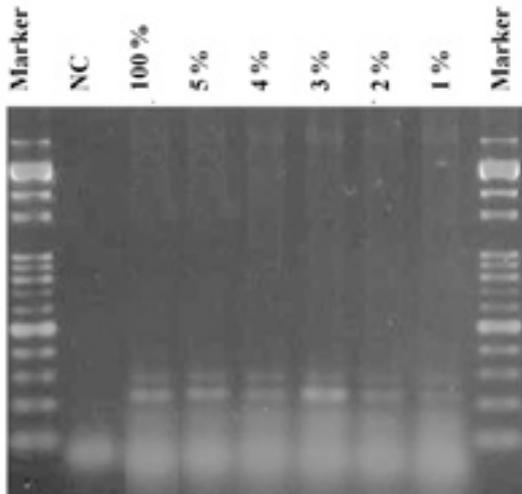


Figure 3. Detecting bovine and porcine-derived MBM mixture in feeds by duplex PCR. Lane 1, 8: 100 bps ladder markers; Lane 2: negative control; Lane 3-8: MBM mixture 100, 20, 10, 5, 2 and 1%, respectively

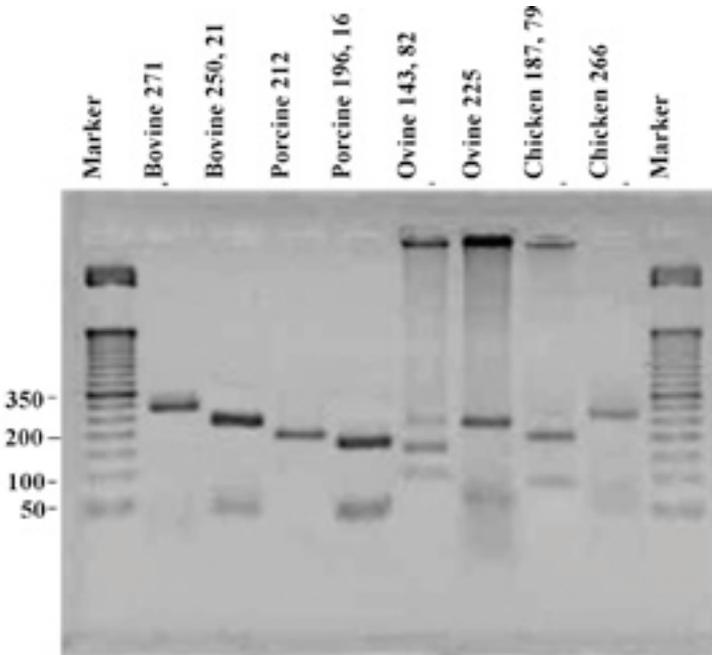


Figure 4. Results of PCR-RFLP analysis. The PCR products were digested with *HphI*-*MnlI*-*SspI*, and *HindIII* restriction enzymes. Lane 1,10 100bps ladder markers; Lane 2: bovine 271 bps; Lane 3: bovine 250,21 bps; Lane 4: porcine 212 bps; Lane 5: porcine 196, 16 bps; Lane 6: ovine 143, 82 bps; Lane 7: ovine 225 bps; Lane 8: chicken 187, 79 bps; Lane 9: chicken 266 bps

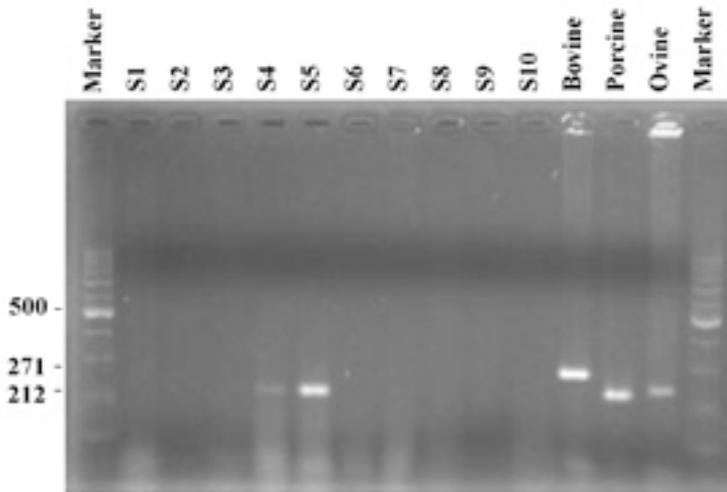


Figure 5. The MBM prevalence survey in ruminant's feeds. Marker: 100 bp DNA ladder markers; S1-S10: Sample No. from 1 to 10 that collected from commercial ruminant feeds. S4 and S5 have positive result in visible gel

TABLE 2  
Results of MBM contaminated ruminant feeds survey during year of 2001-2002 in Taiwan

Species	Positive / Samples	Total prevalence, % (bovine + porcine, %) <sup>a</sup>
Bovine	24/277	24/277 = 8.7%
Porcine	19/277	19/277 = 6.9%
Ovine	ND	
Chicken	ND	

<sup>a</sup> total prevalence: there were 5 samples contaminated both of bovine and porcine derived MBM simultaneously

ND: non-detected

chelex-100 method, because it generated better DNA quality for PCR detection based on a 1% MBM inclusion study (100 vs 67%) (Figure 5). Boom et al. (1999) modified a DNA extraction procedure by addition of alpha-casein to lysis buffer to improve the quality of extracted DNA. Therefore, there is room to improve DNA extraction technique for animal feed products in order to improve DNA quality.

There were many methods developed for meat original species identification, one is enzyme linked immuno-sorbent assay (ELISA), which is a less reliable method for heat-treated products and rendered MBM because soluble muscle proteins degrade quickly under high pressure and high temperature rendering process (Hoffmann, 1996). Furthermore, the detection capability for immunological methods depend on antibody specificity, which is difficult to establish one with low cross-reactivity among closely related species.

Nucleic acid based analysis had widely used in many fields, and become more and more popular for differentiation and identification of feed or food adulterants (Tartaglia et al., 1998; Partis et al., 2000). The advantages of DNA-based analysis are manifold, first is the ubiquity of DNA from all cell type of an individual contains identical genetic information independent of the origin of the samples. Secondly, the information content of DNA is more abundant compared to proteins due to degeneracy of the genetic codes. Thirdly, DNA is a rather stable molecule that renders the extraction and analysis of DNA from many different type of samples feasible.

A detection method based on mtDNA can improve the sensitivity further because each cell has only a set of genomic DNA in the nucleus, but bearing with several copies of mtDNA. mtDNA have a characteristic of high mutation rate, because they are located in cytoplasm and easier to be attacked; there is poor corrective replication of polymerase and lack of proof-reading system in mitochondria. Since mtDNA expressed in different species or genus have their evolution specificities, we can identify individual species by studying mtDNA. There are approximate 1000 mitochondria in a cell and 10 copies of mtDNA per mitochondrion,  $10^4$  copies of mtDNA are available per cell, and just one copy for genomic DNA. Therefore, it is more efficient to detect species-specific DNA by using mtDNA than genomic DNA.

## CONCLUSIONS

Our results showed that there were 6.9 - 8.7% of ruminant feeds contaminated with MBM in Taiwan. The contamination maybe intentionally supplementation or due to carry over occurred in feed mixer after mixing monogastric animal feeds, which occasionally supplement with MBM for protein sources. In current study, the result revealed that PCR method can provide a quick, reproducible and sensitive method to detect MBM supplemented in feeds, even the MBM origin species.

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## STRESZCZENIE

### **Specyficzny tkankowo PCR jako metoda wykrywania mączki mięsno-kostnej w paszy dla przeżuwaczy**

Odkryte w 1986 w Wielkiej Brytanii bydłce gąbczaste zwyrodnienie mózgu (BSE) prawdopodobnie zostało spowodowane dodawaną do paszy mączką mięsno-kostną (MBM) pochodzącą od zainfekowanych chorobą scrapie owiec. Istniejące zagrożenie przeniesienia infekcji na ludzi spowodowało konieczność precyzyjnej kontroli jakości MBM dodawanych do paszy dla przeżuwaczy.

W pracy wykorzystano reakcję PCR do identyfikacji bydłczego, świńskiego, owczego i kurczęcego MBM w dawkach dla zwierząt. Izolację DNA z mączek mięsno-kostnych przeprowadzono na specyficznych kolumnach, a do reakcji PCR użyto czterech par primerów obejmujących wysoce konserwatywne regiony mitochondrialnego DNA (mtDNA) i pozwalające na uzyskanie specyficznych sekwencji DNA dla bydła (271 bp), świni (225 bp), owcy (212 bp) i kurczęcia (266 bp). W celu potwierdzenia specyficzności uzyskanych produktów PCR poddano je trawieniu enzymami restrykcyjnymi: HphI, MnlI, Ssp i HindIII i stwierdzono polimorfizm długości uzyskanych fragmentów restrykcyjnych.

Czułość metody pozwala na wykrycie już 1% MBM w paszy dla zwierząt przeżuwających.