Note

Development of PCR Primers for the Detection of Porcine DNA in Feed Using mtATP6 as the Target Sequence

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In Japan, PCR identification of species-specific, animal group-specific and plant DNA is employed as part of the audit program to ensure compliance with the feed ban in place for the control of bovine spongiform encephalopathy (BSE). Since October 2001, animal proteins other than dairy proteins, egg proteins and gelatin have been prohibited to be used in feed for ruminants. Meat-and-bone meal (MBM) derived from poultry, pig and/or fish is allowed to be used in feed for poultry, pigs and fish. Porcine MBM is permitted in feed for domestic animals other than cattle since April 2005. Given the fact that pigs and cattle are the two major sources of MBM in Japan, the identification of porcine DNA with high specificity and sensitivity has become increasingly important to ensure that MBM products are free from ruminant materials. Two PCR primer sets (PPA8 and PPA6) were newly designed using mtATP8 and mtATP6 as the target sequences, with relatively short amplification sizes. PPA8 and PPA6 were able to specifically detect porcine DNA with the detection limits of 0.01 and 0.001 of porcine MBM in feed, respectively. PPA6 was superior to PPA8 in terms of detection of DNA damaged/fragmented during rendering procedures. The PCR method using these primer sets is registered as the official analytical method for feed in Japan.

Key words: animal feed; meat-and-bone meal; PCR; porcine DNA; primer

Introduction

Bovine spongiform encephalopathy (BSE) is a progressive and fatal disease that affects the central nervous system of cattle. BSE was first confirmed in the United Kingdom (UK) in 1986, and subsequently reported in several other countries in the 1990s. Meat-and-bone meal (MBM) contaminated with a scrapie-like agent is considered to be the vehicle of BSE infection1). Immediately after the detection of the first case of BSE in Japan in September 2001, the Ministry of Agriculture, Forestry and Fisheries (MAFF) prohibited the use of all animal protein (mammalian, poultry and fish protein, excluding dairy products, egg products and gelatin) in feed for ruminants. Meat and bone meal (MBM) contaminated with a scrapie-like agent is considered to be the vehicle of BSE infection1). In May 2008, the feed ban was amended again to allow the use of porcine and poultry MBM in feed for fish.

To ensure the proper implementation of the feed regulations, an audit program composed of on-site inspections of premises involved in feed manufacture, handling, storage or use and taking of feed samples at these premises has been implemented by the Food and Agricultural Materials Inspection Center (FAMIC) under the instructions of the MAFF. The samples are subjected to a range of laboratory tests conducted by the FAMIC for detection of prohibited animal proteins using microscopic, ELISA, and PCR methods for their identification.

PCR can be used for detection of DNA derived from specific species with high sensitivity by using a specific primer set. However, the heat and denaturing treatments applied during the manufacture of MBM may damage the DNA, thus affecting the sensitivity of this method.

The FAMIC has successfully developed primer sets for the detection of mammalian, ruminant, bovine, chicken, fish and plant DNA, which are registered as official primers in the Official Methods of Feed Analysis
in Japan\textsuperscript{1,2,4-6}. The PCR method is of fundamental importance for the detection of prohibited animal-derived material in feed. Furthermore, given the fact that pigs and cattle are the two major sources of MBM in Japan, and to reduce the risk of obtaining a false negative result (for example, due to failure of ruminant DNA extraction) the Official Method of Feed Analysis requires the verification of porcine DNA extraction. This is important, because the starting material used to make MBM is usually highly (heat) processed and DNA extraction from such material is difficult and not always successful.

The chosen target sequences for PCR amplification, namely mitochondrial DNA (mtATP8 and mtATP6), have relatively high degrees of variation among vertebrates\textsuperscript{7}. Similar sequences are not found in plants\textsuperscript{8}. As a result, many primers have been developed using these regions\textsuperscript{9,10}. There are many mitochondria in a somatic cell, and many copies of mtDNA in each mitochondrion. Because of its compact circular shape, mtDNA is relatively stable and thus highly suitable for DNA detection in heat-treated materials. Nevertheless, mtDNA might be damaged/fragmented during the MBM rendering procedure\textsuperscript{4,7}. Recently, a specific detection method for porcine DNA using real-time PCR equipment was reported by Tanabe, et al.\textsuperscript{11}. While this method is rapid, it requires expensive real-time PCR instruments or specific probes. In the present study, we developed two primer sets (PPA8 and PPA6) for detection of porcine DNA in feed using conventional PCR. To detect porcine DNA fragmented or damaged by rendering procedures, we used mtATP8 and mtATP6 as the target sequences, with relatively short amplification sizes. This paper outlines the sensitivity, specificity and other characteristics of these primer sets.

Materials and Methods

Design of PCR primers

PCR primers were designed using the technique described by Kusama, et al.\textsuperscript{3}. In the present study, primers were designed using mtATP8 and mtATP6 as the target sequences to amplify 126 and 83-base pair (bp) DNA fragments, respectively. Table 1 indicates the sequences of the PCR primer sets developed in the present study (PPA8 and PPA6).

Animal and plant mtDNA samples

mtDNA of animals and plants of the following 38 species were extracted from commercial samples: pig (middle Yorkshire, Berkshire, Duroc, Mexican hairless pig, Landrace, miniature pig), cattle (Japanese Black, Angus, Holstein, Japanese Brown), sheep, goat, deer (Nippon Centrails, Nippon Yesoensis), horse, rabbit, mouse, rat, whale, poultry, Japanese quail, duck, sardine, anchovy, jack mackerel, yellowfin tuna, bonito, saury, salmon, cod, righteye flounder, rainbow trout, nippor, horsehair crab, shrimp, squid, short-necked clam, and corn. Human DNA was extracted from hair root.

MBM samples

Samples of porcine MBM, porcine and poultry MBM (MBM of pig and chicken origins), chicken meal and fish MBM were obtained from rendering plants in Japan. The rendering plants in Japan use hyperbaric or atmospheric pressure systems, of which the latter is dominant, to produce MBM. One of the seven MBM samples (MBM2) used in this study had been produced using a hyperbaric process and the other six samples were produced by an atmospheric pressure process (MBM1 and MBM3-7).

Feed samples

Formula feed for cattle that was free from porcine-derived materials was obtained from commercial sources. The feed contained corn (61%), grain sorghum (7%), flour (5%), defatted rice bran (2%), wheat bran (5%), corn gluten feed (8%), soybean meal (3%), rapeseed meal (5%), molasses (1%), calcium carbonate and salt (2%) and a mix of vitamins and minerals.

DNA extraction and PCR amplification

mtDNA was extracted using an extractor CT kit (Wako, Osaka, Japan) based on the alkaline extraction procedure described by Kusama et al.\textsuperscript{3} PCR amplification was performed in a 20-\textmu L reaction volume containing 2-\textmu L (approximately 20 ng) of extracted DNA, 0.4-\textmu mol/L primer set, 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2-\textmu mol/L each primer set and a mix of vitamins and minerals.

Table 1. PCR primers for detection of porcine mtDNA in feed

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplification size</th>
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<tbody>
<tr>
<td>PPA8-F</td>
<td>ATCTACATGATTTAGATTACAAATTAC</td>
<td>126 bp</td>
</tr>
<tr>
<td>PPA8-R</td>
<td>CTATGTTTTGAGTTTTGAGTTCA</td>
<td></td>
</tr>
<tr>
<td>PPA6-F</td>
<td>CTCACCTATTGTCACCTAGTT</td>
<td>83 bp</td>
</tr>
<tr>
<td>PPA6-R</td>
<td>GAGATTGTGCCGGTTATTAATG</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} PPA8-F is referred to as pig 5-3 in the Official Methods of Feed Analysis.
\textsuperscript{b} PPA8-R is referred to as pig 32-2 in the Official Methods of Feed Analysis.
\textsuperscript{c} PPA6-F is referred to as pig 5-6 in the Official Methods of Feed Analysis.
\textsuperscript{d} PPA6-R is referred to as pig 3-6 in the Official Methods of Feed Analysis.

\textsuperscript{1} Notification No. 14729 (Apr. 1, 2008), Food Safety and Consumer Affairs, MAFF, Japan (2008).
Calif.), 200 \mu\text{mol/L} \text{ dNTP mix, 1.5 mmol/L MgCl}_2, \text{and 1\times PCR Gold Buffer. The reaction were performed in a Gene Amp PCR System PE 9700 (Applied Biosystems), with the following cycling conditions: after an initial heat activation at 95°C for 9 min, 45 cycles were programmed as follows: 92°C for 30 s, 55°C 30 s, 72°C 30 s (PPA6) or 92°C for 30 s, 60°C 60 s, 72°C 60 s (PPA8) and final extension at 72°C for 5 min. The PCR products were separated by electrophoresis through 2.5% agarose gel and stained with ethidium bromide.}

**Results and Discussion**

**Specificity**

Figure 1 shows the amplification of the DNA fragment of 83-bp by PPA6. PPA6 amplified porcine DNA only. No amplification was observed from non-target species or feedstuffs. No amplification was observed from MBM of cattle, poultry or fish origin (data not shown). PPA8 had the same specificity (data not shown). These results indicate that PPA8 and PPA6 have a high specificity of PCR amplification from a wider range of feedstuffs than the porcine specific primer sets previously reported\(^9,^{10,12-14}\).

**Sensitivity**

The sensitivity of PCR amplification with primer sets PPA8 and PPA6 was examined with test samples containing 10.0, 1.0, 0.1, 0.01, 0.001 and 0.0001 ng of porcine mtDNA per 20 \mu\text{L} of PCR solution. Both PPA8 and PPA6 were able to detect porcine DNA with a detection limit of 0.0001 ng (data not shown). We also examined the sensitivities using formula feed and chicken MBM containing 0.1, 0.01, 0.001 and 0.0001% of porcine MBM. Figure 2 shows that the detection limits of PPA8 and PPA6 primer sets in formula feed were 0.01 and 0.001 ng, respectively. The detection limits of PPA8 and PPA6 in chicken MBM also were 0.01% and 0.001%, respectively (data not shown). Thus, the sensitivity of PPA6 was equal to, or higher than that of the porcine specific primer sets previously reported\(^10,^{12-14}\).

The DNA in porcine MBM is usually damaged by heat or other treatments during the rendering procedure. The degree of damage varies depending upon the rendering procedure adopted by each plant. Figure 3(A) shows the sensitivity of PCR amplification from MBM samples produced by different rendering procedures using PPA8 and PPA6. Lanes 1–4 and 5–8 were PCR products of mtDNA extracted from MBM samples 1 and 2 shown in (A), respectively; Lane M: 20-bp ladder marker; Lane P: positive control; Lane N: negative control (water).
detect damaged/fragmented DNA effectively, we applied these primer pairs to seven MBM samples. PCR products of two samples (MBM1 and MBM2) are shown in Fig. 3(B). Both PPA8 and PPA6 clearly detected porcine DNA in MBM1. The PCR products of the other five samples (MBM3–7) were also clear (data not shown). PPA6 clearly detected porcine DNA in MBM2, while PPA8 detected it weakly.

These results indicate that the two primer sets are capable of detecting porcine DNA with equal specificity and that PPA6 can detect porcine DNA with a higher sensitivity than PPA8. This is probably because PPA6 is better able to detect damaged/fragmented DNA than PPA8. Given the fact that various rendering procedures are used in Japan, PPA6, which is capable of detecting porcine DNA irrespective of the degree of damage/fragmentation, is particularly useful. It is important to note that the PCR thermal cycle conditions used for amplification with PPA6 are identical with those used for the amplification of DNA using official primers targeting mammals, ruminants, chicken, fish and plants. This enables operators to manipulate the amplification process more efficiently, particularly when there are DNA samples of many species to amplify. In addition, PPA6 has the advantages of high specificity and sensitivity, without requiring a real-time PCR machine.

The new primers are registered as official primers in the Official Methods of Feed Analysis.

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References