EVALUATION OF THE USEFULNESS OF PCR METHOD FOR THE DETECTION OF PROCESSED ANIMAL PROTEIN IN FEEDINGSTUFFS

ANNA WEINER AND KRZYSZTOF KWIAZONEK
Department of Hygiene of Animal Feedingstuffs, National Veterinary Research Institute, 24-100 Pulawy, Poland
kwiatekk@piwet.pulawy.pl

Received for publication July 18, 2008

Abstract

The aim of this paper is to present the results of comparative evaluation of the usefulness of PCR and microscopic methods in the detection of processed animal protein in feedingstuffs. Out of 127 samples of feedingstuffs examined by microscopic method 82 (64.57%) were found to be positive. In comparing the results obtained by microscopic examination and PCR technique, it was found that both methods agreed in 67.7% giving 44 positive and 42 negative results. The rest results (32.3 %) were not coincident. In the validation study, the limit of the detection for PCR was determined on 0.2% for beef and pork meat and bone meal (MBM) and 0.1% for poultry MBM. Analysing results received with the help of the microscopic method and PCR technique it is possible to state that the molecular biology methods can at present, be used as a supplementary method.

Key words: feedingstuffs, processed animal proteins, microscopic method, PCR.

Bovine spongiform encephalopathy (BSE) is a progressive degenerative disease that affects the central nervous system of cattle. It is generally accepted that BSE infection of cattle is caused by feedingstuffs containing processed animal proteins (PAP) contaminated with prions (6, 11-13, 18, 36). In the past, feed materials of animal origin were considered as appropriate ingredients in compound feedingstuffs. Such feedingstuffs had a suitable nutritional value because animal by-products provided a high amount of fat as energy source, appropriate level of protein and minerals (Ca and P), and essential vitamins. As a consequence of the BSE crisis, the use of animal-derived meals as a feed material has been banned in the European Community (EC). The first ban prohibiting the feeding of mammalian processed animal proteins to ruminants was introduced by Directive 94/381/EC. Later on, the permanent ban was enforced in the TSE regulations (8, 9, 19, 29). General rules regarding the safe use of animal by-products are laid down by EC Regulation 1774/2002 (28). Enforcing the above-mentioned regulations would therefore require analytical methods, which will be capable of species-specific detection of PAP, especially proteins from pigs, poultry, and ruminants. However, due to various problems such as lack of methods that fulfill the above mentioned criteria, EC Regulation 999/2001 (29) was amended by Commission Regulations 1234/2003 (8) and 1292/2005 (9) introducing a ban on almost all PAP’s used in animal nutrition. It is worth to note that these regulations clearly indicate the need for a reappraisal of the total meat and bone meal (MBM) ban once new and more specific control methods are available. Currently, the official control method in the EC is based on the detection of constituents of animal origin in animal feedingstuffs by microscopic analysis (7). The EC legislation defines basic guidelines for the identification of animal particles. Fine structures originating from either terrestrial animals or fish are visible during microscopic examination at various magnifications. Main visible characters of animal origin that might be present in feedingstuffs are bone fragments. Additionally, cartilage, hair, feather filaments, eggshells, fish scales, gills, and muscle fibres may also be present and can be detected with the microscopic method. Bone fragments are very persistent particles so that MBM with its high contents of morphological characters is identified by microscopy even after sterilisation at 133°C and 3 bar for 20 min in the rendering process (7, 12, 23, 28, 34).

The need for alternative analytical approaches has prompted numerous studies (3, 22, 24, 32). The application of biomolecular techniques, which tend to be more sensitive and applicable even to high-processed products, has gained increasing interest. Several studies have dealt with the application of PCR for the detection of bovine tissue in animal feedingstuffs (3, 21, 22, 32). In other studies (2, 3, 21, 24), a species-specific PCR has been developed to identify bovine, porcine, and avian tissues in MBM.

In the present work, a specific PCR-based procedure previously optimised in the laboratory for the
detection of animal constituents in feedingstuffs was used in parallel with microscopic technique based on Directive 2003/126 (7, 30) for the detection of animal materials. The sensitivity and usefulness of both techniques were discussed in the light of the results obtained.

Material and Methods

Feed samples. Feed samples were divided into five groups: meat and bone meals (pure ruminant MBM, mixed species MBM, poultry meal, feather meal, fish meal, horn meal, dried pig blood cells), samples of raw plant materials (sunflower, soya, maize, wheat-barley mixture, rape), samples of compound feedingstuffs with a known level of mammalian MBM (2%, 1%, 0.5%, 0.2%, and 0.1 %), and 127 samples of feedingstuffs found as positive (positive control samples) and negative (negative control samples) at the microscopic examination in the laboratory.

For PCR reactions, DNA from feedingstuffs samples was extracted using the NucleoSpin Food (Macherey Nagel) with some modifications. The modifications consisted of increasing the weight of a sample from 200 to 300 mg and increasing the volumes of lysis buffer from 550 µl to 700 µl and proteinase K from 10 µl to 13 µl. The time of lysis was prolonged from 30 min to 3 h. Each DNA amplification was performed in the reaction mixture consisting of DNA template, 1X Taq buffer, 1 U of Taq DNA polymerase, 200 µM of dNTPs, MgCl₂ (5 mM), nucleotide primers, and water to the final volume of 50 µl. The sequences and characteristics of the primers used are shown in Table 1.

PCR reactions were run individually for particular species in a thermocycler (Biometra) under the following conditions: for poultry DNA - initial DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min; the final extension step was done at 72°C for 5 min; for beef DNA - initial DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 66°C for 1 min and 72°C for 1 min; the final extension step was done at 72°C for 5 min; for pork DNA - initial DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min; the final extension step was done at 72°C for 5 min. Then, after staining with ethidium bromide (5 µg/mL) for 1 min and washing in distilled water, the gels were photographed under UV light using the documentation system (Vilber Lourmat). The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

Results

As shown in the Table 2, overall 127 samples, representing 43 samples of feed materials and 84 samples of compound feedingstuffs, were examined for the presence of PAP with use of microscopic method.

Among 43 samples of feed materials, 21 (48.84%) were found positive and contained PAP from terrestrial animals. Out of four samples of compound feedingstuffs for ruminants, two samples contained PAP of terrestrial animal. Moreover, as it appears from the data presented in the Table 2, among 80 samples of compound feedingstuffs for non-ruminants, the presence of PAP of terrestrial animal origin was detected in 59 (73.75%) samples. Overall, processed animal protein presence was detected by microscopic method in 82 (64.57 %) out of 127 feedingstuffs samples.

In the next stage of work, validation of the elaborated PCR procedure was done and the performance criteria for the method were established. It was shown that the limit of detection for this method was 0.2% for beef and pork MBM and 0.1% for poultry MBM (Fig. 1). The established and validated procedure was used for further comparative studies.

Fig. 1. MBM of poultry. Amplicons obtained in the PCR test. M - marker, N - negative control (water), P - positive control (reference material of poultry). Lanes 1, 2-feedingstuffs with MBM (0.1%), 3, 4-feedingstuffs with MBM (0.2%), 5, 6-feedingstuffs with MBM (0.5%), 7, 8- feedingstuffs with MBM (1%), 9, 10-feedingstuffs with MBM (2%).

In the further studies performed, it was shown that out of the total number of 127 samples examined by PCR, cattle DNA was detected in 25 (19.69%) samples, poultry DNA in 31 (24.41%) samples, and pig DNA in nine (7.09 %) samples. Cattle DNA was detected in 16 samples of feedingstuffs for non-ruminants, in one for ruminants, and in eight samples of feed materials (for example: bone meal, organic dicalcium phosphate). Pig DNA was detected in one (2.33%) sample of feed material and in eight (18.60%) samples of feedingstuffs for non-ruminants. Poultry DNA was identified in seven (16.28%) samples of feed materials and in 23 (28.75%) samples of feedingstuffs for non-ruminants and in one (25.0%) sample of feedingstuffs for ruminants. In five (3,94%) samples, DNA of three species (cattle, swine, and poultry) was detected.

These were four samples of feedingstuffs for non-ruminants and one sample of fish meal. In three samples of feedingstuffs for non-ruminants and one sample of fish meal there was identified DNA of cattle and poultry origin. In two samples of compound feedingstuffs for pigs, DNA of cattle and swine origin was detected.
Table 1
Sequences and characteristics of the primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Species</th>
<th>Sequence (5’→3’)</th>
<th>Size of amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCHF</td>
<td>Gallus gallus</td>
<td>TGAGAACTACGAGCACAAC</td>
<td>183</td>
<td>6</td>
</tr>
<tr>
<td>MCHR</td>
<td>Meleagridis meleagridis</td>
<td>GGGCTATTGAGCTACTGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L8129</td>
<td>Bos taurus</td>
<td>GCCATATACTCTCTTGGTGACA</td>
<td>271</td>
<td>32</td>
</tr>
<tr>
<td>H8357</td>
<td></td>
<td>GTAGGCTTGGGGAATAGTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPOF</td>
<td>Sus scrofa</td>
<td>CTATCAAGAATATCCACCACA</td>
<td>290</td>
<td>6</td>
</tr>
<tr>
<td>MPOR</td>
<td></td>
<td>ACATTGTGGGATCCTTCTAGGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2
Number of samples of feed materials and compound feedingstuffs examined by microscopic method and PCR test for the presence of processed animal proteins

<table>
<thead>
<tr>
<th></th>
<th>Number of samples examined</th>
<th>Number of positive samples by microscopic method</th>
<th>Number of positive samples by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number</td>
<td>beef (%)</td>
</tr>
<tr>
<td>Feed materials</td>
<td>43</td>
<td>21 (48.84)</td>
<td>8 (18.60)</td>
</tr>
<tr>
<td>Compound feedingstuffs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for ruminants</td>
<td>4</td>
<td>2 (50.00)</td>
<td>1 (25.00)</td>
</tr>
<tr>
<td>for non-ruminants</td>
<td>80</td>
<td>59 (73.75)</td>
<td>16 (20.00)</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>82 (64.57)</td>
<td>25 (19.69)</td>
</tr>
</tbody>
</table>

Table 3
Comparison results of feedingstuff analysis with microscopic method and PCR technique

<table>
<thead>
<tr>
<th></th>
<th>Microscopic method</th>
<th>PCR test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Positive</td>
<td>44 (53.66)</td>
<td>42 (93.33)</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comparing the results obtained by microscopic method and PCR technique, it was found that both methods agreed in 67.7%, giving 44 positive and 42 negative results (correct results). It means that 32.3% of the results were not coincident (incorrect results), among which 38 were false negative and 3 false positive (Table 3).

Discussion

In the European Community, microscopic evaluation is currently the only accepted method for the detection and characterisation of PAP’s in animal feedingstuffs by this method, and it allows to detect contamination at the request level of 1g/kg with negative and positive results (7, 23, 25, 27, 29, 30). This method is predominantly focused on the presence and characteristics of bone fragments, and other structures, e.g. feathers, and may provide circumstantial evidence of the respective animal types. Recent research works are concentrated more on the identification of bone fragments at the level of classes (mammal versus bird versus fish), supported by image analysis of bone characteristics (25, 27). The method is time-consuming, laborious, requires skill, and incurs considerable cost and delay. The results obtained depend on the experience of the microscopist (14-16).

The application of molecular techniques, which tend to be more sensitive and applicable even to heat-processed products, has gained increasing interest (3-5, 17, 31-33). Several studies have dealt with the application of PCR for the detection of bovine tissue in animal feedingstuffs (3, 22, 32). In PCR studies performed on test samples fortified with processed animal protein, limit of detection (LOD) was determined.
and ranged from 0.1% for poultry to 0.2% for beef and pork MBM. It means that results received in the presented paper are comparable to the scores of German (35) and Belgian (14) researchers. The PCR method described by Tartaglia et al. (32) allowed for the detection of the addition of 0.125% bovine material into fodder. A possibility of receiving correct results independently of the presence or absence of the characteristic morphological structure elements is an additional advantage of the PCR method, since the examination is based on the detection of the mitochondrial DNA, which is present in every cell (20).

It should be pointed out that by PCR technique, 127 samples of different animal feedingstuffs samples were analysed. Out of the overall number of 82 positive samples in microscopic method, the presence of the processed animal protein originating from terrestrial animals was detected and confirmed with PCR method in 44 (53.66%) cases. The low sensitivity of the applied PCR method compared to the microscopic one can be caused by low mass of the sample taken for the analysis (below 1 g), which can be insufficient for detecting low concentrations (0.1%) of processed animal protein (34). Examinations done by different authors (4, 5) are pointing out that heating of the meat tissue at the temperature above 100°C can cause spontaneous fragmentation of DNA, which is the most important barrier to obtain credible results. Boom et al., (1) are informing that inhibitors appearing in feedingstuffs can bind the particles of silica in the last stage of cleaning process of DNA, which perhaps can be a reason of not always correct results obtained in the examination of processed animal protein with the method described by Tartaglia et al., (32).

Moreover, it should be added that with the microscopic method, detection of even a little addition of MBM in feedingstuffs is rather easy. In special cases, the usage of various methods in order to get the more detailed information would be advisable (10, 11). For example, applying the PCR technique is sometimes necessary to determine the kind of the origin of MBM. Moreover, PCR is a technique, which would supplement the results of microscopic observation considering the elements devoid of the characteristic morphological structure (16). Both methods are characterised by very high sensitivity. In case of the PCR technique, this sensitivity was high in the examinations of fortified samples and studies should be continued in order to improve detectability in the samples tested. It seems that mass of the sample used plays a significant role in the examinations. It should be underlined that for the examination with the microscopic method, at least 5 g sample should be used, but with the PCR technique, only 0.4 g is sufficient. Additionally, a regular mixing of feedingstuffs is of great significance and provides their homogeneity at the stage of production at the manufacturing company. It is possible to formulate such speculation taking into consideration the acquired experience and achieved results during analysis of fortified samples. These fortified samples were prepared in the amount of 10 g with regular mixing of elements, which facilitated the detection. Then, even when 0.3 g of mixed sample was used, the detection of the added MBM was possible.

The analysis of the results received with the help of the microscopic method and PCR technique has shown that methods of the molecular biology at present can be used as supplementing tool, according to suggestions of different researchers (20, 26, 34). It results from the fact that the microscopic method gives credible results also in highly processed feedingstuffs but with retained morphological structures, even when the genome material is highly degraded. On the other hand, the PCR technique offers an alternative of analysis of samples with genomic material, even in the case of the lack of typical and characteristic morphological structures.

References


