Testing animal feed for the presence of ruminant DNA using the official real-time PCR method

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Abstract. Feed has become liable to testing for the presence of ruminant DNA as part of the eradication process of transmissible spongiform encephalopathies. For safety reasons, by the so-called total feed ban, meat and bone meal has been excluded from the diet of food chain animals for years. However, changes in EU regulations that led to relaxation of this ban began from mid-2013 with the introduction of animal proteins derived from poultry and pigs in feed for aquaculture. The EU published an approved PCR method for determining the species of animals from which the ingredients originate. The EU also validated a real-time PCR protocol for detection of prohibited ruminant DNA. As a result of harmonization with the EU legislation, in 2016, amendments to the Serbian TSE regulations were published and an identical method of feed control prescribed. In the same year, this procedure was implemented by the Institute of Veterinary Medicine of Serbia and accredited to the required standard, so adequate feed control started. The aim of this paper is to present the results of the first 50 animal feeds or feed materials tested for the presence of ruminant DNA using the official real-time PCR method. A high percentage (54 %) of the 50 feeds/feed materials studied contained ruminant DNA.

1. Introduction
Processed animal proteins (PAP) for use in animal nutrition are largely obtained as by-products of dairies, slaughterhouses and the fish processing industry. These feedingstuffs are characterized by a high percentage of proteins (over 50 %, or some types even close to 80 %). They all possess significant biological value due to their ideal amino acid composition. Consequently, especially in developed countries, PAP was often used in the feeding of highly productive animals. However, after the outbreak of bovine spongiform encephalopathy (BSE), commonly known as mad cow disease and diagnosed in the UK in 1986, it was found that this prion spread via feed, if the infectious ruminant proteins were processed into meat and bone meal (MBM). Eradication started immediately and one of the most important measures was to pass regulations to exclude entry of these nutrients into the food chain [1,2].

EU Regulations 999/2001 [3] and 1234/2003 [4] prohibit the application of PAP, which include the various types of MBM, for all farm animals entering the human food chain, except fishmeal to non-ruminants. Also, regulation 1774/2002 [5], repealed by regulations 1069/2009 [6] and 142/2011 [7], prescribed general guidelines for the safe use of by-products of animal origin and prohibited the use of proteins originating from the same species in animal nutrition. Such strict measures were introduced because the incriminated ingredients can potentially cause prion infections not only in animals, but also indirectly in humans, through their consumption of food of animal origin.
On the other hand, a total ban on MBM in the diet of farm animals, although very successful in terms of eradication of transmissible spongiform encephalopathies (TSE), is a waste of highly valued proteins, which has brought great losses in the economic and environmental sense. Annual production of animal by-products from the meat, milk and eggs supply in the EU is about 17 million tons. Therefore, not only because of the nutritional importance, but also in terms of sustainability, re-introduction of PAP in the diet of farm animals would have numerous advantages. Also, it is estimated this step would provide an annual profit of about € 350 million [8].

The control system in Serbia, in comparison with the countries of the European Union, was somewhat more complex, with differences in regulations and applied preventive measures. In 2001, amendments to the regulation on quality and other requirements for feed [9] officially banned animal by-products in the diet of ruminants for the first time, while animal by-products were still allowed in diets for monogastric animal species. Identical measures were prescribed by regulation on the quality of feed [10], which came into force on 1st of May 2010. However, the regulation on determining, diagnosis and preventing transmissible spongiform encephalopathies [11], from 1st of April 2011, introduced a total ban of MBM for all farm animals, equivalent to the ban in Europe. Such a partial feed ban, although preferable for economic and nutritional reasons, enabled cross contamination of feed for ruminants with prohibited ingredients. Therefore, according to article 110 of the Veterinary Law in Serbia [12], feed producers were obligated to separate lines for ruminant feed, or to dispose of MBM and fish meal. Control of feed for the presence of MBM and fishmeal using classical microscopy, as prescribed by EU regulations, started in 2006. From 2011, this monitoring has become a part of the annual state program.

However, changes in EU regulations and relaxation of the ban began from mid-2013 with the introduction of animal proteins derived from poultry and pigs in feed for aquaculture. In addition to classical microscopy, EU Regulation 51/2013 [13] approved a PCR method for detecting the species of animals from which the ingredients originate. The EU Reference Laboratory for Animal Proteins in Feedingstuffs also validated a real-time PCR protocol for detection of prohibited ruminant DNA. As a result of harmonization with the EU legislation, on the 1st of April 2016, amendments to the Serbian TSE regulation [14] were published, and an identical method of feed control was prescribed. In the same year, this procedure was implemented by the Institute of Veterinary Medicine of Serbia and accredited to SRPS ISO/IEC 17025 standard, so adequate feed control started. The aim of this paper is to present the results of the first 50 animal feed samples tested for the presence of ruminant DNA using the official real-time PCR method.

2. Materials and methods
Altogether, 50 samples of MBM (declared as pure poultry, pig or without the declaration) or fish feed and similar products of animal origin were tested. The applied method was the one prescribed by Serbian TSE regulation [14] and described in detail on the web site of the EU Reference Laboratory for Animal Proteins in Feedingstuffs (EURL-AP). It is presented in the form of an official SOP and protocol [15].

3. Results and discussion
Following the European legislation, regular feed analysis started in Serbia soon after implementation of the new real-time PCR protocol. Results of the first 50 tests, grouped according to feed/feed material type, for the presence of ruminant DNA are presented in Table 1.

| Type of animal feed/material | Number (%) of positive feeds/materials | Number of negative feeds/materials |
|-----------------------------|----------------------------------------|-----------------------------------|
| Poultry MBM                 | 18 (62)                                | 11                                |
| Pig MBM                     | 0                                      | 1                                 |

Table 1. Presence of ruminant DNA in 50 animal feeds or feed materials from Serbia
From the data given in Table 1, it is obvious that numbers and percentages of animal feeds/feed materials containing ruminant DNA was high. As we observed with the start of microscopic examination [2], it takes some time for new laws to be fully accepted. Besides that, the PCR method is very sensitive and detects extremely low levels of contamination. It is an efficient way to copy small numbers of DNA segments, and theoretically, one copy can be amplified. Intentional adulteration or attempted fraud is even easier to discover. Therefore, when this is understood and accepted, a downward trend should be expected for the presence of ruminant DNA in animal feeds/feed materials where it is not allowed. Those feeds/feed materials with confirmed positive results showing they contain ruminant DNA can only be used in pet and fur animal feeds.

The introduction of PCR as the official method and the validation of a PCR assay for the detection of ruminant DNA in feed allowed the re-authorization of non-ruminant PAP in feed for aquaculture. It is important to emphasize that according to the present operational scheme, PCR should be used in combination with light microscopy for analysis of feed or feed material for aquaculture. The operational protocol for the analysis of feed or feed material intended for farmed animals other than aquaculture animals and fur animals (e.g. feed for ruminants, pigs, poultry, horses, rabbits,…) includes only the light microscopy method [16]. For this reason, excellent knowledge of the current regulation and SOP is very important for choosing the method to be applied. Otherwise, the wrong choice of method can give a result that is unusable in practical circumstances. Moreover, it can lead to wrong conclusions and can cause problems (RASFF notifications, recall and withdrawal of batches, financial losses and, ultimately, possible health consequences) in the later phases of control or use of feed.

The next steps will be use of poultry PAP for pigs and pig PAP for poultry, as validation of adequate PCR protocols that would allow efficient control are on the horizon. Due to interference of authorized animal ingredients (e.g. fats, blood products, dairy products) with PCR results, additional analytical approaches will probably be needed. In addition to its own research, in 2014, the EURL-AP initiated an international laboratory network to investigate and develop alternative techniques, such as aptamers, mass spectrometry and ELISA. The most promising method is probably mass spectrometry [17]. Recently the identification of proteins and peptide biomarkers allowing the detection of PAPs by mass spectrometry produced very interesting results, but efforts must be continued and the journey to validation and implementation by the control laboratories is long.

Furthermore, the implementation of a third category of animal material in addition to terrestrial, i.e., the terrestrial invertebrates, is proposed [18]. According to novel data, insects are a promising feed and food protein source, but future research needs to provide some solutions before they can be widely utilized in food and/or feed [19]. It is important to fill the existing analytical gaps and to detect the species of interest rapidly. In that regard, the most promising approach today is real-time PCR.

The design of laboratory methods should serve for monitoring authenticity, control of labels/declarations and detection of fraud [20]. Especially when considering the future relaxation of the ban, however, the possibility of cross-contamination remains the biggest threat. Strict avoidance of these undesirable cases (unauthentic, undeclared, unlabeled, fraudulent or cross-contaminated feeds) will remain an obligation for all participants in the food chain.

4. Conclusion
Testing of feed for the presence of ruminant DNA using the official real-time PCR method is no longer new. Over time and by understanding the need for these analyses, participants in the food chain should begin to take advantage of timely detection of unauthorized ingredients. For full effect, laboratories play an extremely important role. Their knowledge of current regulations and SOPs is
crucial for choosing the method to be applied. Otherwise, mistakes at this stage can produce results that are not usable in practical circumstances. Moreover, they can lead to wrong conclusions and cause many problems in later phases of control or use of feed. Therefore, it is essential that feed producers and feed material importers ensure full compliance with legislation and use a functional system of hazard analysis and critical control points. To prevent cross-contamination or intentional adulteration, regular and routine control of raw materials and complete feeds is extremely advisable.

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