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Incidence of horse meat in processed food on B&H market

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Abstract

Following the "horse meat scandal" in 2013, European Union countries have conducted official control of EU market and unraveled food fraud which implicated a number of processed food products and food businesses. Five years after the breakout of the scandal, no official information on market surveillance in Bosnia and Herzegovina is available. Therefore, 73 randomly selected meat products from retail were collected and analyzed for the presence of horse DNA. Horse DNA was detected in 21 products (28.77%). Particularly disturbing for B&H consumers is high proportion of sujuk samples positive for horse DNA (46.15%) with lower incidence among the products of small manufactures. Also disturbing is the finding that 71.43% of the products that contain horse DNA were produced in B&H. According to our data there is a requirement for stricter surveillance of both import and internal market.

Keywords

"horse meat scandal", PCR marker, sujuk, meat species, food fraud, adulteration

Introduction

In 2013, Food Safety Authority of Ireland (FSAI) announced the results of meat authenticity survey thus starting the "horse meat scandal", as it is now known. The DNA based survey exposed traces of horse DNA in 10 out of 27 samples of frozen beef burgers offered in retail stores. The quantitative analysis estimated that one sample contained 60-100% of equine DNA compared with 5-30% of bovine DNA.

In the following months, food fraud of European scale was revealed which implicated a number of food products and food businesses. Sharp decline in ready meals sector and renewed interest in traditional butchery products are some of the outcomes of the scandal (O'Mahony, 2013).

Although the scandal was described as rather the issue of mislabeling than the one of food safety, there is also a health concern about the presence of phenylbutazone (PBZ) in meat obtained from racing horses which finds its way into the supply chain. Phenylbutazone is banned for use in animals bread for human consumption; it is also the most frequently used non-steroidal anti-inflammatory (NSAID) drug in horses. While horses are not raised for food in the USA, over 60 million pounds (>27 million tonnes) of horse meat is exported annually

and it was established that a number of horses that received PBZ were sent for slaughter (Dodman et al., 2010).

Labelling regulations are put in place in order to protect the consumers' rights. According to the EU Regulation 1169/2011 consumers need to have essential information on the composition of the product, preparation methods, storage, the producer etc. Therefore, specific and sensitive methods for identification of animal tissues are developed in order to ensure that food products are accurately labelled. Intensive processing of animal meats before obtaining final product may cause problems in identification of animal species. When processing interferes with analytical procedure the possibility that food adulteration remains undiscovered increases (Schlumpberger, 2004; Martín et al., 2008).

In order to test food authenticity and avoid fraudulent practices it is necessary to provide accurate, specific and sensitive analytical methods suitable for detection and identification of meat species in processed food products. Many methods based on the analysis of species specific compounds (such as proteins and DNA) have been developed for this purpose. Protein methods are mostly based on immunological assays, chromatography and electrophoretic techniques (Berger et al., 1988; Armstrong et al., 1992; Gallardo et al., 1995). However, protein based methods are less efficient when it comes to products that are heat-treated and highly processed because of denaturation of meat proteins (Hoffman, 1996).

Methods based on DNA analysis are much more suitable because it is possible to amplify specific DNA fragment after heat treatments even if partial degradation of DNA sequence occurs (Martín et al., 2008).

PCR (polymerase chain reaction) based methods for species identification in foodstuffs rely on the specific amplification of one or more DNA fragments so it is very important to select DNA sequences that show the most difference between different animal species (Rodriguez et al., 2004; Kesman et al., 2010). DNA markers of conserved regions of mitochondrial and nuclear DNA are mostly used for species identification. The attempts to discredit the *FSAI*'s report were thwarted because it was based on well validated DNA based analytical methods (O'Mahony, 2013).

Five years after the "horse meat scandal" the data on the incidence of horse meat in processed food on the market in Bosnia and Herzegovina (B&H) are deficient. Thorough search yields only media announcements alleging that a number of analyzed samples did contain undeclared horse meat but no official report can be found. Therefore we conducted a study in order to (a) select, optimize and validate highly specific and sensitive DNA marker for detection and identification of horse DNA (b) to explore the incidence of horse meat in processed food products on the market of B&H.

Materials and methods

We collected 73 random processed meat products over two year period (2013-2014; 22 pâté, 6 salami, 16 sausage, 13 sujuk, 11 luncheon meat, 5 frankfurter) off the shelves of retail centers in our country. None of the samples had horse meat listed among the ingredients. The sampling included both domestic (41) and imported products (32). DNA extraction was performed using modified CTAB Soltis protocol (Doyle & Doyle, 1987; Cullings, 1992). This method is routinely used for DNA extraction from plant material but was adapted for processed food in our laboratory. The modification includes maceration of 1 g of processed food product with 5 ml of buffer (CTAB, ß-mercaptoethanol, polyvinylpyrrolidone) whereupon samples are incubated and extraction procedure continued according to the original protocol. The extracted DNA was subjected to spectrophotometric analysis in order to measure DNA concentration and test for purity. To test whether the extracted DNA contains amplifiable DNA we used PCR to amplify fragment of mitochondrial cytochrome B gene (*cyt b*, 195 bp; Pfeiffer et al., 2004) in beef products and chromobox-helicase-DNA-binding gene (*CHDZ*; Griffiths et al., 1998) for chicken/turkey products. Detection and identification of horse DNA was performed according to Kesmen et al. (2007) method with modifications. The primer pair CTATCCGACACACCCAGAAGTAAAG (*ATPase8*) and GATGCTGGGAAATATGATGATCAGA (*ATPase6*) was used for amplification of 153 bp fragment of mitochondrial ATP synthase subunit 8 and ATP synthase subunit 6 genes. This fragment is highly specific and its small size allows for amplification

from highly degraded DNA. PCR amplification reactions were performed in total volume of 10 µl. Instead of using separate components of PCR reactions we used RedTaq ready mix (2X; Sigma Aldrich) which contains 3 mM $MgCl_2$, 0.4 mM dNTP mix and 0.3 u/µl TaqDNA polymerase. For optimization of PCR reactions we used 1X RedTaq (Sigma Aldrich), 0.5 mM and additional 1 mM of $MgCl_2$ (the total of 2 mM $MgCl_2$), 0.3 µl of each primer and 40 ng template DNA. As a template for optimization of PCR reactions and subsequent PCR positive control we used DNA extracted from blood of *Equus caballus*. Extraction of DNA from blood was performed according to Miller et al. (1988). After an initial step of denaturation at 95°C for 2 minutes, 35 thermal cycles were carried out – melting at 95°C for 30 seconds, annealing at 47.4°C-62.6°C (in 2°C intervals) for 30 seconds, extension at 72°C for 30 seconds and final elongation step at 72°C for 4 minutes using Eppendorf Mastercycler Gradient. Optimised PCR method was used for detection and identification of horse DNA in processed food products. Amplification products were electrophoretically determined using 1.5% agarose gel stained with Midori Green (Nippon Genetics).

Results and Discussion

Modified CTAB Soltis protocol yielded DNA of sufficient purity and quantity from all 73 samples despite quite variety of processed food products. The level of degradation was high, as expected, considering that all the sampled products were intensely processed. Concentration of DNA measured spectrophotometrically ranged between 0.0228 and 0.5419 µg/µl. Values of A260/A280 ratio were >1.7 for 60 (82.19%) analyzed samples. Such values indicate low protein contamination and respectively sufficient purity of DNA extracts for downstream analyses.

Amplification of *cyt b* was successful in 60 analyzed samples. However, 13 samples tested negative for the presence of *cyt b*. Even though all the samples that tested negative were chicken/turkey/porcine, positive *cyt b* amplification was expected because of dairy ingredients (whey, milk proteins, milk, powdered milk). However, it is important to consider that the method was not validated for dairy products. Out of 60 products that tested positive for *cyt b*, 28 were declared as beef so the positive result of amplification was expected. Some of the other food products that tested positive contain whey and milk proteins or adipose and connective tissue that may possibly be of bovine origin. However, the list of ingredients on 16 food products lists nothing that could explain positive amplification of *cyt b*. The cause of positive amplification may be related to the production process in industries that produce both beef and poultry products.

Amplification of *CHDZ* region was successful in 30 out of 38 products listed as chicken/turkey. Out of 8 products that tested negative 5 products were chicken/turkey while 3 products contained poultry compounds as secondary. Analysis of horse specific amplicons generated in PCR reactions using the same thermal protocol and different $MgCl_2$ concentrations (2mM and 3mM) showed that specific amplicons were generated using both concentrations. However, using 3 mM $MgCl_2$ more unspecific amplicons were obtained while PCR reactions with 2 mM $MgCl_2$ resulted with clear bands indicating better specificity. The results of gradient PCR reactions for different annealing temperatures showed that the best efficiency was obtained 55.5ºC-60.1ºC. For further analyses 58ºC annealing temperature was used. Considering that optimized PCR protocol differs from the original Kesmen et al. (2007) method applied it to DNA extracts of porcine tissue and bovine DNA standard. The optimized PCR method did not show cross reactivity with porcine or bovine DNA.

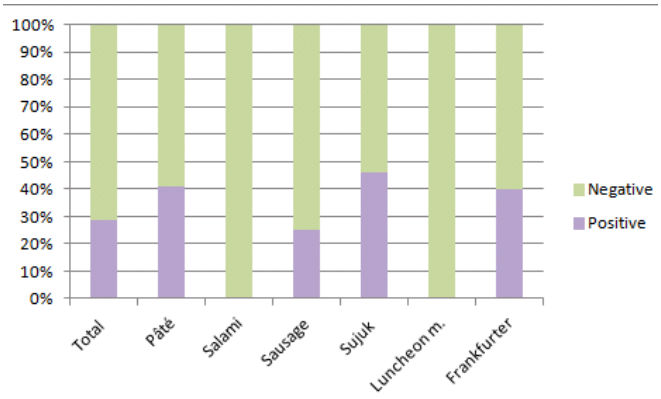

Figure 1. Fraction of samples positive for horse DNA per food category

For analysis of food products that went through intensive processing the fact that DNA molecules are more stable than proteins is crucial. Food processing

Table 1. Incidence of horse DNA in the analyzed samples according to the food type

	Total	Pâté	Salami	Sausage	Sujuk	Luncheon m.	Frankfurter
Positive	21	9	0	4	6	0	2
Negative	52	13	6	12	7	11	3
Total	73	22	6	16	13	11	5

includes heat treatment and other processes such as marinating and fermentation which makes protein methods unsuitable.

Methods relaying on DNA analysis give more accurate results when it comes to species identification (Meyer et al., 1995). The results of Kesmen et al. (2010) study showed that PCR method is suitable for identification of horse DNA in sujuk products regardless of additives used and the fermentation process. The obstacles faced during identification of animal tissues in processed meat products are largely overcome using methods based on PCR thus enabling detection of meat species that are not preferred by consumers but can be incorporated into different meat products.

In our study horse DNA was detected in 28.77% (21 food products) of the 73 analyzed food products even though horsemeat was not listed in the ingredients list (Table 1, Figure 1). Out of 21 samples that tested positive, amplification was quite efficient for 11 samples (5 sujuk, 4 pâté, 1 sausage and 1 frankfurter) indicating that horse DNA presence is not the result of accidental contamination. Food products like sujuk are potentially risky because different meat species possibly of low quality can be used in its production (Kesmen et al., 2010). Frankfurters and pâté contain minced tissues which makes this type of food products suitable for possible fraudulent adulterations. According to our results 11 samples of pâté and frankfurters, out of 27 analyzed, contained horse tissue.

The results of sujuk testing may be particularly interesting for B&H consumers since that is widely favored product. Of the 13 tested sujuk samples 6 (46.15%) were positive for horse meat. Interestingly though, most of the negative sujuk samples were produced in small family manufactures. It suggests that the risk of accidental contamination is lower in facilities with lower diversity of products. Also, for small manufactures sujuk is a mean to market lower quality meat rather than the main product. In addition to intentional replacement of quality beef with undeclared horse meat of dubious quality for financial gain, possible sources of contamination may be inadequate control over raw materials and substitution of meat with different animal tissues.

It is particularly distressing that 15 positive samples were products of B&H which accounts for 71.43% of the samples that tested positive (Table 2, Figure 2). Also, it makes 36.58% of all tested samples produced in B&H. Considering the extent of import of raw materials from Hungary, Germany, Poland, Romania, the countries which detected undeclared horse meat in various products during official control in 2013 (Stanciu et al., 2013) it is evident that the "horse meat scandal" has rolled over into B&H market as well. The percentage of imported samples positive for horse DNA is also notable – 18.75% and suggests a need for strengthening the capacities of control system, including cross-border control, in order to detect potential adulterations of the different parts of the food supply chain.

Table 2. Incidence of horse DNA in the analyzed samples according to the place of origin

	Total	B&H	Import
Positive	21	15	6
Negative	52	26	26
Total	73	41	32

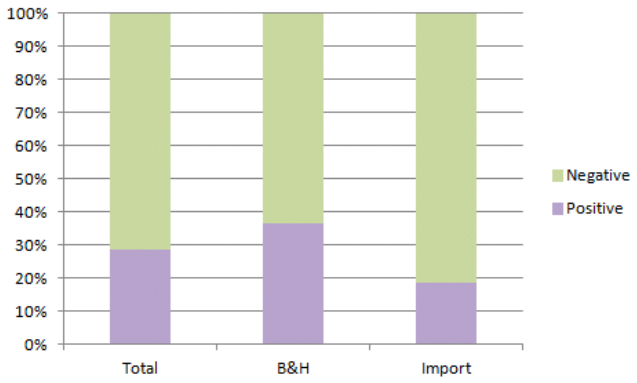

Figure 2. Fraction of samples positive for horse DNA according to the place of origin

Conclusions

Optimized DNA extraction method showed to be efficient for obtaining DNA of sufficient quality and quantity from processed food products. The optimized species specific PCR method used in our study allows detection of small amounts of horse tissue in meat products without unspecific amplification. The results demonstrate the utility and applicability of the used method for detection of undesirable meat adulterations in foodstuffs which are expected to have fragmented DNA due to intensive processing. Detection of undeclared horsemeat in 28.77% analyzed food products indicates the necessity of more intensive market surveillance and strengthening the capability of the control system in order to enforce labelling regulations and protect consumers' rights.

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