Review of testing for foreign horse and pig DNA in meats in Croatia

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Abstract. Several years after the food industry scandal when horsemeat was found in products sold in Europe as beef products in 2013, Croatia began testing food for the presence of foreign protein. For the time being, these tests are not part of routine monitoring, but the result of examining the situation on the market in the city of Zagreb. Namely, in recent years, central Croatia has been trying to establish itself as a tourist destination, and Zagreb hosted hundreds of thousands of tourists from all over the world before the COVID-19 pandemic. The eating habits of the various groups that came to Zagreb were different, and the larger hotel chains recognized the seriousness of the services and sought help to ensure that the food offered was consistent with their declarations and would not conflict with religious requirements. One of these requirements was the testing for foreign proteins such as horse and pork in foods where they were not declared. Although horse and pork are safe for human consumption, they are not part of the eating habits in all countries. The Dr. Andrija Štampar Teaching Institute for Public Health introduced methods for detection of horse and pig DNA in food samples.

1. Introduction

The general principle of food law is to provide consumers with a basis to enable them to be properly informed when choosing the food they consume and to prevent actions that may mislead consumers. The European Union therefore adopted Regulation (EU) No 1169/2011 [1]. The objectives of Regulation (EU) No 1169 are a high level of protection of the health and interests of consumers, with special emphasis on health, economic, environmental, social and ethical circumstances [1].

Europe was rocked by a food industry scandal in 2013 when horse meat was found in products sold as beef products, according to the declaration. Undeclared or misdeclared other meats such as pork were also found in the products. In some products, horsemeat accounted for 100% of total meat. The affair began on January 15, 2013, when it was revealed that horse DNA had been found in frozen burgers sold in Irish and British supermarkets. Shortly after this announcement, the scandal spilled over into many European countries, where products were also found to contain undeclared horse meat in various meat products. Numerous falsely declared products have been withdrawn from supermarket shelves across Europe [2].

Although horse meat is safe for human consumption, it is not part of the eating habits of all European countries. The falsification of declarations and fraud in the food industry usually have financial reasons, as prices for quality meat are high. Horse meat is much cheaper than other types of meat in some countries. There is a similar problem with pork. Indeed, there are large groups of people who do not want to eat pork for religious reasons [3].
Proper labelling of the type of meat contained in food products is important for economic, safety, legal and health reasons. Misdeclared meat is of questionable origin and there is no guarantee that it is safe for consumption. Some people do not consume the meat of certain animal species due to religious customs and laws [4]. In Croatia, food labelling is regulated by the Consumer Information Act (NN 56/13) and Regulation (EU) No 1169/2011 on informing consumers about foods. Nevertheless, the legislation in the Republic of Croatia regarding the examination of the presence of foreign proteins is very open for the time being. Namely, according to Art. 6 of the Regulation on Meat Products NN 62/2018, a meat product that has a prominent animal species in its name must contain at least 75% of the meat derived from that animal species, calculated on the total amount of meat used in the production process [4,5].

This formulation certainly does not satisfy sensitive groups of people regarding eating habits. The question arises as to what amounts of foreign protein such groups would tolerate. Due to such questions, the British Food Standard Agency has developed guidelines for the limits of acceptability of the amount of foreign protein in the product. Currently, a foreign protein level of 0.1-1% is considered acceptable. Back in 2014, the recommendation to the food industry was that this would be technically feasible in terms of good manufacturing practice and acceptable to most consumers. It is considered that a limit of up to 0.1% foreign protein should not be reported but should be monitored regularly. Where control samples show 0.1-1% foreign protein, the reasons for this presence should be investigated and corrective action taken. For products containing 1% or more of a foreign protein, this should be declared or the recall of such products should be encouraged [6].

Testing for the presence of foreign proteins, such as those from horse and pig, can be performed by various methods based on immunological assays, chromatography, and other chemical methods [7]. Most of these methods are limited due to their sensitivity and easy denaturation of proteins by a rise in temperature [8]. Methods based on DNA analysis, such as polymerase chain reaction (PCR), are more robust as well as sensitive and specific. Compared to proteins, DNA is a more stable and resistant molecule. It is resistant to various processes used in the food industry such as food processing at high temperatures and pressures, presence of other chemical compounds, etc. PCR can, therefore, analyse processed foods, as the DNA molecule is not destroyed by food processing such as case for proteins. The PCR method can also be used to successfully identify certain types of meat present in meat mixtures [7,8].

The Dr Andrija Štampar Teaching Institute for Public Health offers services for testing foods for the presence of DNA of horse and pig origin. These DNAs can be successfully detected in various food samples, from fresh meat (e.g. mixed minced meat), to various meat products such as sausage and salami and even in ready-to-eat meals. [9] First, DNA must be isolated from the sample. Then, all necessary amplification reagents are added to the isolated and purified DNA. If foreign DNA is present in the sample, it is amplified, which is noticeable by an increase in fluorescence [10].

2. Materials and Methods
In the last five years from 2016-2020, a total of 43 samples of different types of sausages and salamis were tested for the presence of DNA derived from horses and 51 samples were tested for the presence of DNA derived from pigs. Table 1 shows the number of samples tested.

| Year of testing | Horse DNA | Pig DNA |
|----------------|-----------|---------|
| 2016           | 3         | -       |
| 2017           | 10        | 10      |
| 2018           | 10        | 10      |
| 2019           | 10        | 21      |
| 2020           | 10        | 10      |
2.1 DNA extraction

DNA was extracted according to the protocol using the foodproof Sample Preparation Kit III (Biotecon Diagnostics). The extraction buffer was added to 200 mg of homogenized sample in 2 ml microcentrifuge tubes and vortexed for 30 seconds. Proteinase K (80 µl) was added to the suspension containing sample and extraction buffer. The mixture was incubated at 72 °C for 30 min and the tube was mixed 2-3 times by inverting the tube during incubation. Then centrifugation was done at 12000 x g for 10 min to remove the insoluble material. The supernatant was transferred to a new microcentrifuge tube with 400 µl Binding Buffer and 200 µl isopropanol and mixed gently but thoroughly by pipetting up and down.

The mixture (650 µl) was pipetted into the upper reservoir of a combined filter-collection tube and centrifuged at 5000 x g for 1 min. The collection tube was discarded and filter tube was transferred to a new collection tube. The remaining mixture was added to the same filter-collection tube and centrifuged again at 5000 x g for 1 min. The flow through and collection tube were discarded, and the filter tube was added to a new collection tube. Wash buffer (450 µl) was added to the upper reservoir and centrifuged at 5000 x g for 1 min. The flow-through was discarded and the collection tube was reused for a new step in which 450 µl wash buffer was washed and centrifuged at 5000 x g for 1 min. The flow-through was discarded again and the collection tube reused for a centrifugation of 10 sec at maximum speed to remove residues of wash buffer.

The dried column was transferred to a clean 1.5 mL microcentrifuge tube. Pre-warmed (70 °C) elution buffer (200 µl) was added to the glass fibre fleece and left at room temperature for 5 min to ensure the elution buffer was completely absorbed. Finally, the column was centrifuged at 5000 x g for 1 min to elute the purified DNA, which was used directly or stored at -20 °C for further analysis.

2.2 Polymerase Chain Reaction (PCR) amplification

The extracted and purified DNA was amplified by real-time PCR (PicoReal 24, Thermoscientific). Amplification of DNA was performed with Thermo Scientific PikoReal Software 2.2. The assay is a qualitative duplex real-time PCR, which means that the detection of specific genes and internal control is performed simultaneously using specific primers marked by fluorescent colours. Specific genes for porcine and horse animals and the internal control were detected by FAM (porcine/horse specific gene) and HEX/VIC (internal control) detection channels.

For Porcine detection Lyokit, 25 µL extracted and purified DNA, 25 µL negative control (PCR-grade H2O), and 25 µL positive control (control template) were added into each PCR tube which already contained lyophilized reagents. The PCR cycler program included pre-incubation in two steps: 4 minutes at 37 °C and 10 minutes at 95 °C; 5 seconds at 95 °C and 60 seconds at 60 °C.

For Horse Species Detection Kit, a reaction mixture was prepared by combining 4µL of primer/probe mixture, 10 µL of real-time PCR mastermix, 6 µL of extracted DNA sample into each PCR tube, so the reaction volume for each tube was 20 µL. Positive and negative controls were used. The program included pre-incubation at 95° for 10 minutes and amplification in two steps: 15 seconds at 95 °C and 40 seconds at 61°C.

3. Results

Figure 1 shows the meat products in which the DNA of horse origin was examined according to year. From 2017 to 2019, tests were carried out on samples of permanently cured meat products declared as 100% pork or beef of domestic or imported origin. In 2017, two meat products were positive for horse DNA. During 2018 and 2019, no horse DNA-positive meat products were found. Samples of domestic and imported origin of brands from individual retail chains and domestic producers were examined. Horse DNA was not found in any of the samples. In 2020, semi-durable meat products such as various salamis, hot dogs, and the like were tested. Only in one sample was DNA of horse origin found in traces.
**Figure 1.** Presence of horse DNA in meat products labelled as pork or beef during five years

Figure 2 shows the meat products in which DNA of pig origin was examined according to year. The tested meat products were semi-durable meat products. The figure shows a slow increase in the number of samples positive for pig-derived DNA.

**Figure 2.** Presence of pig DNA in meat products during four years

Figure 3 shows the results of cleaning control in a company that exports its products to countries where halal is practiced.
Figure 3. Cleaning processes control
In 2019, some Halal certified companies decided to control their cleaning processes to achieve greater trust of customers in their products. Production lines in smaller companies cannot separate products according to individual types of meat. Therefore, cross-contamination of the products can occur. Sometimes in products declared as 100% non-pig type of meat, traces of unwanted pig DNA were found. Various cleaning and disinfection procedures were tried, until testing showed no traces of unwanted pig DNA. Unfortunately, during 2020, these controls abated due to the pandemic.

4. Conclusion
Foods that are produced or imported before being placed on the market must meet specified standards. For this reason, regular controls are needed to make sure that consumers are consuming exactly the type and quality of meat that is declared. Meat companies have or want to obtain certificates that are mandatory in some countries due to ethical rules, religions or eating habits, and the companies do not rely only on their national regulations or EU regulations, but go a step further and control the presence of foreign proteins in their production.

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