Molecular diagnostic test systems for meat identification: A comparison study of the MEAT 5.0 LCD-Array and innuDETECT Assay detection methods

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Abstract

The aim of the study was to compare the efficiency, sensitivity and reliability of the MEAT 5.0 LCD-Array and innuDETECT Assay detection kits in identifying selected animal species. Samples were taken from the femoral muscles of six animal species (turkey, chicken, cattle, pig, sheep and goat), and six variants of binary meat mixtures were analysed at 18 different concentration levels of addition. The MEAT 5.0 LCD-Array test was able to detect 0.1% of other meat additions in two meat mixtures and 0.5% in four meat mixtures. The innuDETECT Assays were able to detect the addition of 0.1% of other meat in three meat mixtures, 0.5% in two mixtures and 1% in one meat mixture. Subsequently, these methods were applied in practice to 136 samples of various products taken from commercial food networks. By performing extensive monitoring, we identified 60 products in which one to three species were detected besides what was present on the product label. Nine products were contaminated with pig DNA. Two products that the MEAT 5.0 LCD-Array kit identified as positive for the presence of pig DNA were not confirmed by the innuDETECT Pork Assay kit. We recommend these methods of analysis to comprehensively monitor the presence of animal species in food samples, regardless of the degree of heat treatment or mechanical processing, as a tool to detect food adulteration.

Quantification, detection kit, animal species, products

Meat adulteration cases and related traceability problems have garnered much more attention due to customer requirements and administrative responsibility. Therefore, it is important to develop efficient systems and methods with high sensitivity for rapid detection and identification of specific sources of meat samples (Xu et al. 2018). In the case of meat and meat products, the authentication should pay particular attention to confirming whether higher-quality raw material has been replaced wholly or in part by less valuable components or whether weight-increasing additives, e.g. water, fat, fat substitutes (starches, gelatine, fibre, etc.), or proteins from other sources, e.g. from soybeans and the like (Suhaj and Kováč 2000) have been used. In meat, the most frequent cases of adulteration have been by replacing expensive meat with less valuable meat from other animal species. Examples include the use of meat of domestic swine (*Sus scrofa domestica*) instead of wild boar (*Sus scrofa scrofa*) in game meat products (Fajardo et al. 2008; Spielmann et al. 2018). The identification of meat species by DNA is often preferred over their identification by proteins (Vallejo-Cordoba et al. 2005; Montowska and Pospiech 2010; Alikord et al. 2018). Böhme et al. (2019) state that DNA-related techniques to detect food adulteration include polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), DNA microarray and next-generation

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sequencing (NGS), DNA metabarcoding, DNA-barcoding high-resolution melting (Bar-HRM), loop-mediated isothermal amplification (LAMP) and digital droplet PCR (ddPCR). The use of a DNA microarray is an alternative genetic approach to simultaneous detection of various plant and animal species as well as bacteria present in a sample of interest. It offers several advantages, specifically the identification of unreported and unknown animal species present in the meat sample that were introduced by unintentional contamination or deliberate adulteration of meat products (Kemp et al. 2005; Azuka et al. 2011). RT-PCR can be used in the routine detection and quantification of animal species (Kesmen et al. 2009).

The aim of this study was to compare DNA-based methods (MEAT 5.0 LCD-Array kit and the innuDETECT Pork Assay kit) of identifying animal species and apply them to various products obtained from markets.

Materials and Methods

Sampling

Meat samples were obtained from a grocery store, and mixtures were prepared using muscle tissue from a pig (*Sus scrofa*), sheep (*Ovis aries*), goat (*Capra hircus*), turkey (*Meleagris gallopavo*), chicken (*Gallus gallus*) and cattle (*Bos taurus*, *Bos bison*). Mixtures of meats were prepared using Blender 8008 (Waring Commercial, Torrington, Connecticut, USA) to a final weight of 100 g. Each piece of muscle tissue of the animal species was ground separately. The grinding was accomplished by passing the tissue through a small hand grinder and blender. Mixing was done in 5 cycles for 3 min. In the meantime, the mixing vessel was placed in the refrigerator at 8 °C for 10 min. Samples were taken from the ground pure muscle tissue and isolated. Binary mixtures designed to verify the potential of the compared methods were prepared by dilution of DNA in the laboratory. The initial concentration was 20 $ng \cdot \mu l^{-1}$, from which we subsequently made the mixtures. To confirm the efficiency/use of the methods, we analysed 136 commercial samples. These samples were collected from local markets in Slovakia and coded appropriately. The food products included seven ingredients for confectionery, 25 meat spreads, 75 liver pâté products, 15 dry fermented sausages, 14 cooked hams and patties.

DNA was extracted from a 50-mg ground sample of pure muscle tissue and also from purchased meat products using the Maxwell 16 Tissue DNA Purification Kit and Maxwell 16 system (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions. Extractions were replicated × 3. The amount of DNA in each sample was quantified using a Quantus fluorimeter (Promega, Madison, Wisconsin, USA). DNA solutions were stored at -18 °C until further use. All DNA extracts used for both analyses had the same concentration. Positive and negative controls were included in each analysis for both combined animal species of binary mixtures.

Analysis using the MEAT 5.0 LCD-Array kit

Each MEAT 5.0 LCD-Array chip (Chipron, Berlin, Germany) contains 25 species-specific capture probes fixed to each chip. These probes, immobilized as duplicates, allow simultaneous detection of 17 mammalian species (cattle, sheep, horse, goat, camel, buffalo, pig, kangaroo, hare, rabbit, reindeer, roe deer, red deer, fallow deer, springbok, dog, cat) and seven bird species (chicken, turkey, goose, ostrich, mallard duck, Muscovy duck, pheasant) in food preparations. PCR runs were performed in a TOptical Gradient 96 thermocycler (Biometra, Göttingen, Germany). Amplification was performed according to the manufacturer's instructions. Each PCR reaction contained 25 µl of an amplification mixture consisting of 12.5 µlL of × 2 Master mix (including × 10 PCR buffer, 1.5–2.0 mM $MgCl_2$ and 10 mM each dNTP mix and Taq Polymerase 5 $U \cdot \mu l^{-1}$ (EC 2.7.7.7)), 1.5 mL of primer mix 'MEAT', 6 µl of PCR grade water and 5 µl of diluted DNA sample. Primer mix "MEAT" and × 2 Master mix were supplied in the MEAT 5.0 LCD-Array kit. The cycle regime was set to one cycle for initial denaturation for 5 min at 95 °C, 35 repetitions including denaturation for 30 s at 94 °C, annealing for 45 s at 57 °C and elongation for 45 s at 72 °C. The last step, strand competition, ended the PCR program and took 2 min at 72 °C. To verify the presence of amplified DNA in each sample, electrophoresis on 2% agarose gel was used. LCD array hybridization was performed according to the manufacturer's instructions. During hybridization (at 35 °C, 30 min), labelled PCR fragments were bound to specific immobilized capture probes as dark precipitate at the bottom of each chip and were visualized by a PF3650u LCD-array scanner (PacificImage Electronics, Torrance, California, USA) using SlideReader V12 software (Chipron, Berlin, Germany). Reactions were replicated twice per analysis. The default detection cut-off threshold was a pixel value of 2000 (MEAT 5.0 Manual, version 1-1-2014).

Analysis using InnuDETECT Assay kits

Samples analysed using the MEAT 5.0 LCD-Array Kit were verified by real-time PCR using innuDETECT Assay kits (Analytic Jena, Berlin, Germany). The procedure given for the innuDETECT (chicken, pork, turkey, sheep, goat, beef) Assay was followed according to the manufacturer's instructions. All solutions in the assay

were dissolved before use: 25 µl of the PCR mixture contained 10 µl of × 2 Master mix, 3 µl of Primer/Probe Mix × IC (Internal Control), 1 µl of IC, 5 µl of diluted DNA sample and 1 µl of PCR-grade water. The cycle regime was set to one cycle for initial denaturation for 120 s at 95 °C, 35 repetitions including denaturation for 10 s at 95 °C, annealing for 45 s at 62 °C and elongation for 45 s at 62 °C. The tubes were analysed on a LightCycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler® 2.0 Software 4.1 (Roche Diagnostics, Mannheim, Germany). Reactions were replicated × 3 per analysis. Pork DNA concentration was determined using a standard curve of pure pork DNA after making serial dilutions starting with 100 $ng \cdot \mu l^{-1}$ DNA. Threshold cycle values (Ct) were plotted against logarithms of DNA concentration to generate a standard curve for pork DNA. Linearity was observed in pork DNA in the range of six orders (100–0.001 $ng \cdot \mu l^{-1}$).

Results

In comparing the sensitivity, efficiency and reliability of the detection kits, we have included the six most common types of meat in meat production and processing in Central Europe. In order to evaluate the specificity of the MEAT 5.0 LCD-Array kit and to verify the absence of potential cross-reactivity, pure meat samples for the species were first analysed. Also, the InnuDETECT Assay kit includes an Internal Control, which was added to the PCR reaction as an amplification control.

In the first mixture (Table 1), a combination of chicken and pork in which we added the chicken to the pork in descending order, both detection kits correctly evaluated the presence of pig DNA in the analysed samples. Combining 1% chicken with 99% pork, we found a negative result when using the innuDETECT Chicken Assay kit. From the threshold of a 1% addition of chicken to pork, the reaction failed. The MEAT 5.0 LCD-Array kit did not identify the addition of 0.1% chicken and was assessed as unsuccessful at this concentration step of addition.

Table 1. Results for mixtures of pork and chicken.

Samples	Tested mixtures	Detection kits			
		MEAT 5.0 LCD-Array		innuDETECT Assay	
		pork	chicken	pork	chicken
1	100% chicken meat	-	+	-	+
2	100% pork meat	+	-	+	-
3	90% chicken + 10% pork	+	+	+	+
4	80% chicken + 20% pork	+	+	+	+
5	70% chicken + 30% pork	+	+	+	+
6	60% chicken + 40% pork	+	+	+	+
7	50% chicken + 50% pork	+	+	+	+
8	45% chicken + 55% pork	+	+	+	+
9	40% chicken + 60% pork	+	+	+	+
10	35% chicken + 65% pork	+	+	+	+
11	30% chicken + 70% pork	+	+	+	+
12	25% chicken + 75% pork	+	+	+	+
13	20% chicken + 80% pork	+	+	+	+
14	15% chicken + 85% pork	+	+	+	+
15	10% chicken + 90% pork	+	+	+	+
16	5% chicken + 95% pork	+	+	+	+
17	1% chicken + 99% pork	+	+	+	-
18	0.5% chicken + 99.5% pork	+	+	+	-
19	0.1% chicken + 99.9% pork	+	-	+	-

+ detected; - undetected

Table 2. Results for mixtures of beef and pork.

Samples	Tested mixtures	MEAT 5.0 LCD-Array		innuDETECT Assay	
		Beef	Pork	Beef	Pork
1	100% beef meat	+	-	+	-
2	100% pork meat	-	+	-	+
3	99.9% pork + 0.1% beef	+	+	-	+
4	99.5% pork + 0.5% beef	+	+	+	+
5	99% pork + 1% beef	+	+	+	+
6	95% pork + 5% beef	+	+	+	+
7	90% pork + 10% beef	+	+	+	+
8	80% pork + 20% beef	+	+	+	+
9	70% pork + 30% beef	+	+	+	+
10	60% pork + 40% beef	+	+	+	+
11	50% pork + 50% beef	+	+	+	+
12	45% pork + 55% beef	+	+	+	+
13	40% pork + 60% beef	+	+	+	+
14	35% pork + 65% beef	+	+	+	+
15	30% pork + 70% beef	+	+	+	+
16	25% pork + 75% beef	+	+	+	+
17	20% pork + 80% beef	+	+	+	+
18	15% pork + 85% beef	+	+	+	+
19	10% pork + 90% beef	+	+	+	+
20	5% pork + 95% beef	+	+	+	+
21	1% pork + 99% beef	+	+	+	+
22	0.5% pork + 99.5% beef	+	+	+	+
23	0.1% pork + 99.9% beef	+	+	+	-

(Column group header: Detection kits)

+ detected; - undetected

In the second mixture (Table 2), a combination of pork and beef in which we added the pork to the beef in descending order and vice versa, both detection kits correctly evaluated the presence of beef and pork in the analysed samples. With combinations of 0.1% pork + 99.9% beef and 0.1% beef + 99.9% pork, we found a negative result when identifying pork and beef with the innuDETECT Pork Assay kit. The MEAT 5.0 LCD-Array kit identified the addition of 0.1% in both replicates of the analysis and was assessed as successful over the 18-degree concentration scale.

In the third mixture (Table 3), a combination of turkey and pork in which we added the turkey meat to the pork in descending order, both detection kits correctly evaluated the presence of pig DNA in the analysed samples. Combining 0.5% turkey with 99.5% pork, we found a negative result when identifying the turkey meat with the innuDETECT Turkey Assay kit. From the 0.5% addition of turkey meat to pork, in each of the repetitions, the reaction failed. The MEAT 5.0 LCD-Array kit did not identify the addition of 0.1% turkey meat, as in the first mixture with the chicken, in both replicates of the assay and was therefore assessed as unsuccessful at this concentration step of addition.

In the fourth mixture (Table 4), a combination of mutton and pork in which we added the sheep meat to the pork in descending order, both detection kits correctly evaluated the presence of pig DNA in the analysed samples. With a combination of 0.5% mutton

Table 3. Results for mixtures of pork and turkey.

Samples	Tested mixtures	Detection kits			
		MEAT 5.0 LCD-Array		innuDETECT Assay	
		Pork	Turkey	Pork	Turkey
1	100% turkey meat	-	+	-	+
2	100% pork meat	+	-	+	-
3	90% turkey + 10% pork	+	+	+	+
4	80% turkey + 20% pork	+	+	+	+
5	70% turkey + 30% pork	+	+	+	+
6	60% turkey + 40% pork	+	+	+	+
7	50% turkey + 50% pork	+	+	+	+
8	45% turkey + 55% pork	+	+	+	+
9	40% turkey + 60% pork	+	+	+	+
10	35% turkey + 65% pork	+	+	+	+
11	30% turkey + 70% pork	+	+	+	+
12	25% turkey + 75% pork	+	+	+	+
13	20% turkey + 80% pork	+	+	+	+
14	15% turkey + 85% pork	+	+	+	+
15	10% turkey + 90% pork	+	+	+	+
16	5% turkey + 95% pork	+	+	+	+
17	1% turkey + 99% pork	+	+	+	+
18	0.5% turkey + 99.5% pork	+	+	+	-
19	0.1% turkey + 99.9% pork	+	-	+	-

+ detected; - undetected

and 99.5% pork, we found a negative result when identifying sheep meat with the innuDETECT Sheep Assay kit. With the 0.5% addition of mutton to pork in each of the three replicates, the reaction failed. The MEAT 5.0 LCD-Array kit did not identify the addition of 0.1% mutton in either replicate of the analysis and was unsuccessful at this addition concentration step.

In the fifth mix (Table 5), a combination of goat and pork in which the goat meat was added to the pork in descending order, both detection kits correctly evaluated the presence of pig DNA in the analysed samples. Combining 0.1% goat with 99.9% pork, we found a negative result when identifying goat meat with the innuDETECT Goat Assay kit. Similarly, the MEAT 5.0 LCD-Array kit did not identify the addition of 0.1% goat meat in either of two replicates and was also evaluated as unsuccessful at this addition stage.

In 136 commercial products, manufacturers declared the main ingredient as pork, beef, chicken, duck, goose or turkey meat. Several products were identified that contained animal species (e.g., beef, pork, chicken, turkey, duck, goose, sheep and goat DNA) other than those indicated on the product label. Table 7 shows the percentage of incorrectly labelled samples and the percentage of samples that were contaminated with pork DNA. The aim of the analyses was to identify pork DNA in products. Pork meat is consumed in Slovakia in the largest quantity and it has been the subject of many cases involving food adulteration. In nine products, we detected the presence of pork DNA outside the labelling. Products such as turkey hams and salami, chicken meat paste, chicken burger patties, vegetable spread, salmon and tomato pepper spread were contaminated.

Table 4. Results for mixtures of pork and sheep.

Samples	Tested mixtures	Detection kits			
		MEAT 5.0 LCD-Array		innuDETECT Assay	
		Pork	Sheep	Pork	Sheep
1	100% sheep meat	-	+	-	+
2	100% pork meat	+	-	+	-
3	90% sheep + 10% pork	+	+	+	+
4	80% sheep + 20% pork	+	+	+	+
5	70% sheep + 30% pork	+	+	+	+
6	60% sheep + 40% pork	+	+	+	+
7	50% sheep + 50% pork	+	+	+	+
8	45% sheep + 55% pork	+	+	+	+
9	40% sheep + 60% pork	+	+	+	+
10	35% sheep + 65% pork	+	+	+	+
11	30% sheep + 70% pork	+	+	+	+
12	25% sheep + 75% pork	+	+	+	+
13	20% sheep + 80% pork	+	+	+	+
14	15% sheep + 85% pork	+	+	+	+
15	10% sheep + 90% pork	+	+	+	+
16	5% sheep + 95% pork	+	+	+	+
17	1% sheep + 99% pork	+	+	+	+
18	0.5% sheep + 99.5% pork	+	+	+	-
19	0.1% sheep + 99.9% pork	+	-	+	-

+ detected; - undetected

Discussion

Beltramo et al. (2017) worked with the MEAT 5.0 LCD-Array detection kit with DNA levels ranging from 6.6 to 25 ng·μl^{-1} for raw meat mixtures containing different concentrations of contaminating species. They analysed 17 types of mixtures combining two to four species to identify species specificity. Our results are consistent with their findings. They found that for the meat samples, at the 0.1% level the adulterant species were not always detected in both replicates, as they were at the 0.5% and 1% levels. The kit manufacturer declares a detection limit of < 0.5% (w/w) depending on the sample's processing level (MEAT 5.0 Manual).

All animal species were successfully identified at 0.5% meat addition in all samples using both methods. Using the MEAT 5.0 LCD-Array kit, the 0.1% addition was identified only in the case of adding beef to pork and vice versa. Based on the results obtained by comparing the sensitivity, efficiency and reliability of both detection methods, we can conclude that in these two products was a lower concentration of pork DNA that innuDETECT Pork Assay kit could not detect (0.1%). The results presented in Table 2 show the limits of the innuDETECT Pork Assay kit in determining pork concentration in meat mixtures. Similar results were obtained by Al-Kahtani et al. (2017).

Such small amounts of pork DNA in commercial food products are likely to result from cross-contamination of the production line rather than from deliberate adulteration of food products with pork (Al-Kahtani et al. 2017). In a survey of 42 samples of Turkish meat products, Ulca et al. (2013) found four samples positive for pig DNA. Ali et al. (2014)

Table 5. Results for mixtures of pork and goat.

		Detection kits			
Samples	Tested mixtures	MEAT 5.0 LCD-Array		innuDETECT Assay	
		Pork	Goat	Pork	Goat
1	100% goat meat	-	+	-	+
2	100% pork meat	+	-	+	-
3	90% goat + 10% pork	+	+	+	+
4	80% goat + 20% pork	+	+	+	+
5	70% goat + 30% pork	+	+	+	+
6	60% goat + 40% pork	+	+	+	+
7	50% goat + 50% pork	+	+	+	+
8	45% goat + 55% pork	+	+	+	+
9	40% goat + 60% pork	+	+	+	+
10	35% goat + 65% pork	+	+	+	+
11	30% goat + 70% pork	+	+	+	+
12	25% goat + 75% pork	+	+	+	+
13	20% goat + 80% pork	+	+	+	+
14	15% goat + 85% pork	+	+	+	+
15	10% goat + 90% pork	+	+	+	+
16	5% goat + 95% pork	+	+	+	+
17	1% goat + 99% pork	+	+	+	+
18	0.5% goat + 99.5% pork	+	+	+	+
19	0.1% goat + 99.9% pork	+	-	+	-

+ detected; - undetected

Table 6. List of analysed and contaminated products.

Products	n	Incorrectly labelled [%]	Porcine DNA contamination [%]
Ingredients for confectionery	7	28	14
Meat spreads	25	16	12
liver pâté products	75	22	8
Cooked hams and patties	14	78	21
Dry fermented sausages	15	60	6

identified chicken nuggets containing pork. Demirhan et al. (2012) used real-time PCR and porcine-specific primers for halal authentication of gelatine and found that the minimum level of adulteration detected was 1.0% w/w in marshmallows and gumdrops. Of a total of 32 samples from Turkey, 31 samples were found to be negative. Ran et al. (2016) found porcine DNA in 15 commercial samples (minced meat, braciola, sausage, meatballs) of a total of 42. Sahilah et al. (2012) found that 42 of 113 pharmaceutical capsules they tested contained pig DNA. Another study reported that 65 minced meat samples, 35 meatballs, 50 fermented sausages, 125 pork fermented sausages, 135 sausages, of which 410 were sampled, were determined to have an adulteration ratio of 19.2% (79 samples; Gu̇ns̨en et al. 2006). The verification of the declared composition of the food as indicated on the label is an officially mandatory task to ensure the protection of public health against counterfeiting (Özpinar et al. 2013).

In conclusion, the analysis of foodstuffs revealed an unflattering prevalence of adulteration, which indicates an intensive need for further investigation, development and enhancement of the effectiveness of identification and quantification techniques by combining the examination of several aspects in one analysis. The MEAT 5.0 LCD-Array Kit was shown to be a quick method allowing for accurate determination of the presence of various kinds of meat in products. It could be used as a method for official control of meat and meat products.

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