Detection of meat adulteration: Use of efficient and routine-suited multiplex polymerase chain reaction-based methods for species authentication and quantification in meat products

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Summary

The increase in the extent of meat adulteration is the reason for a need for an effective method for authentication of meat products. DNA-based polymerase chain reaction (PCR) is a well suited alternative for this purpose. Furthermore, the method facilitates quantification of animal DNA in meat products based on the correlation between target copy amounts and cycle numbers in quantitative PCR. We designed and experimentally verified PCR primer systems for identification of beef, pork, horse and poultry (chicken, turkey) meat. Mitochondrial and chromosomal markers were used. The mitochondrial cytochrome b gene was used as a marker for qualitative multiplex endpoint PCR and single-copy chromosomal genes (cyclic-GMP-phosphodiesterase gene for cattle, beta-actin gene for pig, interleukin-2 gene for chicken, myostatin gene for mammals and poultry) were used for multiplex quantitative PCR analyses. The reliability of both methods was confirmed by analysing of mixed samples prepared with or without heat treatment. The methods were then applied to 14 commercially available products typical for the Czech Republic, including sausages or salami. Discrepancies were observed between the DNA analysis and the meat content declared for the tested products, as two of the samples did not correspond to qualitative requirements and other four failed to meet quantitative requirements. The proposed PCR-based methodology was shown to be useful for the disclosure of meat adulteration.

Keywords

DNA; multiplex polymerase chain reaction; authentication; meat

Meat and meat products belong to the most expensive foods and therefore fall into the category of frequently adulterated commodities. Customers are often deceived through the substitution of a high-quality meat with a less valuable kind or incorrect/false labelling of the proportions of components. This unfair behaviour offers profit to the dishonest producer. Meat adulteration is a serious problem on a global scale. The scandal with horse meat in 2013 can be taken as an example of food fraud in Europe, as horse meat was found in meat products labelled as beef in the EU market [1]. Adulterated meat was also delivered to the Czech Republic [2].

Such irregularities may be detected by physical, chemical or biochemical techniques. Nucleic acids, proteins or metabolites are often used as markers. The most used techniques are gas chromatography [3, 4], liquid chromatography [5, 6], near-infrared spectroscopy [7], mid-infrared spectroscopy [8], matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [9], electrophoresis with sodium dodecyl sulphate (SDS) [10], capillary electrophoresis, isoelectric focusing, direct analysis in real time/time of flight mass spectrometry (DART/TOF-MS) [11], enzyme-linked immunosorbent assay (ELISA) [12] and polymerase chain reaction (PCR) [13].

Proteins and DNA are the most discriminative markers for detection of food adulteration. For this purpose, protein analysis can be done in simple format of ELISA, but these methods have

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their own limitations, particularly concerning structural changes of target molecules during heat processing. DNA is more thermostable than proteins and, consequently, DNA-based methods can be used for analysis of heat-treated meat products. In principle, DNA analysis facilitates a very precise identification of animal species in food products. However, while the detection of undeclared meat in food products is a relatively simple matter, its quantification is a complex issue [14, 15].

Molecular-biological methods, in particular various types of PCR with or without a connection to sequencing, have become the main techniques for meat authentication. Quantitative real-time PCR or digital PCR can be used as effective tools for quantification of nucleic acids. While PCR with one pair of primers allows for the analysis of one target sequence, the addition of multiple pairs of primers to the reaction mixture in multiplex PCR makes it possible to analyse multiple parameters in one test tube simultaneously. Multiplex PCR is often used for the detection and quantification of food ingredients and genetically modified organisms in food analysis. The first use of multiplex PCR for meat authentication was published in 1999. MATSUNAGA and colleagues performed simultaneous identification of bovine, pig, chicken, sheep, goat and horse DNA. The mitochondrial gene of cytochrome b was used as a marker [16]. Another similar method for the detection of beef, poultry, fish and pork was described in 2004 [17]. Since then, further multiplex PCR methods for authentication and quantification of DNA from various animals were developed [14, 18, 19].

The objective of the present study was the construction of a detection system for beef, pork, horse and poultry meat based on DNA analysis. The selected species are the most frequently consumed in EU. The developed method was also used to monitor commercial meat products from the market in the Czech Republic.

MATERIALS AND METHODS

Sample preparation

Samples of meat and meat products were obtained from commercial sources in the Czech Republic. Muscle meat of cattle, pig, chicken, duck, turkey, horse, goose, quail, salmon and mackerel were used as meat reference samples. Segments from the internal part of meat, pieces of musculature and meat products were homogenized in a grinder IKA A10 (IKA-Werke, Staufen im Breisgau, Germany), weighed out and stored at –20 °C until required. A human DNA sample was obtained from a buccal sample using a forensic swab (FLOQSwabs Genetics, Brescia, Italy).

Tab. 1. Composition of commercial meat products.

Product	Declaration of meat content on a product label	Declared percentage replacement of chicken/beef/pork meat in a product*		
		Chicken	Beef	Pork
Luncheon meat pork	Pork and beef 70 %, pigskin		100 %	
Spicy salami	Beef 31 %, pork 29 %, lard		52 %	48 %
Pepper sausage	Pork 63 %, beef 12 %, pigskin	–	16 %	84 %
Sausages	Pork 65 %, beef 13 %	–	17 %	83 %
Veal sausages	Veal (minimally 40 %), pork (minimally 34 %)	–	54 %	46 %
Meatloaf	Pork 42%, chicken meat 23%	35 %	–	65 %
Small sausages I	Pork and beef 65 %, lard and pigskin	–	100 %	
Salami RIO EBRO	Pork and beef 80 %, lard, pigskin	–	100 %	
Debrecener sausages	Pork and beef 70 %, lard and pigskin 11 %	–	100 %	
Pate	Lard, pork 10 %, pig liver 9 %, mechanically separated chicken meat 6 %, pigskin	24 %	–	76 %
Hungarian sausage	105 g of pork and 10 g of beef	–	9 %	91 %
Small sausage II	Pork 40 %, lard, beef 10 %, pigskin	–	20 %	80 %
Vienna sausage	Mechanically separated chicken meat 40 %, lard and pigskin, mechanically separated pork meat 8 %, beef	72 %	≤ 14 %	14 %
Luncheon meat	Mechanically separated chicken meat 37%, pork 23 % and 9 % mechanically separated pork, pork fat	54 %	–	46 %

* – conversion which excluded irrelevant proportions like water, spices and other additives [20].

Tab. 2. Sequences of the used oligonucleotide primers and probes.

Meat species	Name	Target	Sequence of primer [5'–3']	Size [bp]	Ref.
Common forward primer	SIM	Cytochrome b (mitochondrial DNA)	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA		[16]
Beef	B		CTA GAA AAG TGT AAG ACC CGT AAT ATA AG	274	[16]
Pork	P		GCT GAT AGT AGA TTT GTG ATG ACC GTA	398	[16]
Chicken, turkey	C		CGT ATT GTA CGT TCC GGC AAG	169	this study
Horse	H		CTC AGA TTC ACT CGA CGA GGG TAG TA	439	[16]
Beef	Bos-PDE-f	Cyclic-GMP-phospho-diesterase (chromosomal DNA)	ACT CCT ACC CAT CAT GCA GAT	104	[22, 23]
	Bos-PDE-r		TGT TTT TAA ATA TTT CAG CTA AGA AAA A		
	Bos-PDE-p		TexasRed:AAC ATC AGG ATT TTT GCT GCA TTT GC:BHQ-2		
Pork	Sus1-F	Beta-actin (chromosomal DNA)	CGA GAG GCT GCC GTA AAG G	107	[22, 24]
	Sus1-R		TGC AAG GAA CAC GGC TAA GTG		
	Sus1-p		VIC:TCT GAC GTG ACT CCC CGA CCT GG:BHQ-2		
Mammals and poultry	MY-F	Myostatin (chromosomal DNA)	TTG TGC AAA TCC TGA GAC TCA T	97	[22, 25]
	MY-R		ATA CCA GTG CCT GGG TTC AT		
	My-p		FAM:CCC ATG AAA GAC GGT ACA AGG TAT ACT G:BHQ-2		
Chicken	ChIn-F	Interleukin-2 (chromosomal DNA)	TGT TAC CTG GGA GAA GTG GTT ACT	135	[23]
	ChIn-R		CTG ACC ATA AAG AAT ACC TAC CG		this study
	ChIn-p		TAMRA:TGA AGA AAG AAA CTG AAG ATG ACA CTG AAA TTA AAG:BHQ-2		[23]

Both homogenized meat blends and mixtures of isolated DNA from animal species (pig, beef, horse and chicken) in various ratios were used as references. Minced meat mixtures were prepared from samples of homogenized muscle meat. Minced meat fractions from pork and beef musculature were mixed to obtain samples of 100 g final weight as follows (in grams): 1:99, 5:95, 10:90, 25:75, 50:50, 75:25, 90:10 and 95:5. Heat-treated meat samples were prepared from homogenized beef, pork, horse and chicken meat containing 2% iodinated salt (Gemma di Mare, Rozzano, Italy). Fifty grams were heated at temperatures of 70, 100, 120 and 180 °C for 30 min in a hot-air oven.

Commercially available meat products were randomly selected in the market. The composition, declared on product label and meat content after conversion, which excluded irrelevant components like water, spices or other additives [20], is shown in Tab. 1.

DNA isolation

DNA was isolated from 200 mg of homogenized samples of meat or meat products, or from the buccal swab, using a cetyltrimethylammonium bromide (CTAB) method according to ČSN EN ISO 21571 [21]. The integrity of the isolated DNA was determined by electrophoresis in 1% agarose gel with staining by Midori Green Advance (Elisabeth Pharmacon, Croydon, United Kingdom). DNA concentration and purity were determined by measurement of absorbancies, at 230 nm, 260 nm, 280 nm and 320 nm, by a NanoPhotometer (Implen, Munich, Germany). DNA was diluted as detailed below with nuclease-free water (Promega, Madison, Wisconsin, USA).

Primers and probes

Primers and probes used in this study are listed in the Tab. 2. All oligonucleotides were obtained from East Port Prague (Prague, Czech Republic). Two primers were newly designed, others were previously reported [16, 22–25]. Limit of detection was determined by calculation of the false negative rate (below 5 %) on 10 replicates as described in verification of analytical methods for genetically modified organisms (GMO) testing [26]. Selectivity was tested *in silico* and experimentally. In case of *in silico* testing, available databases such as Nucleotide database (National Center for Biotechnology Information – NCBI, Bethesda, Maryland, USA), European Nucleotide Archive (European Bioinformatics Institute, Cambridge,

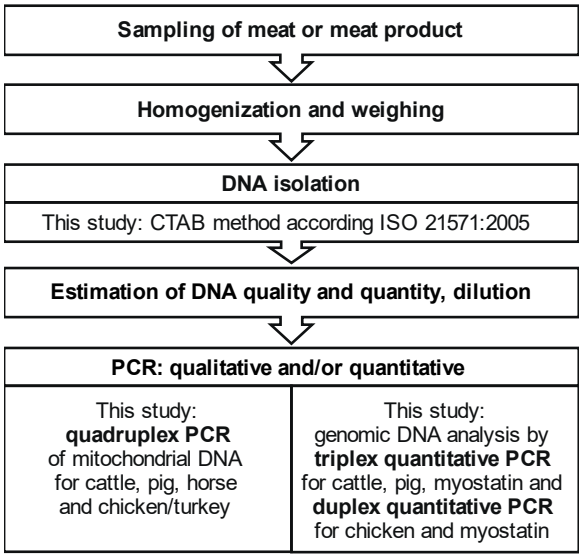

Fig. 1. Workflow for authentication of origin of meat and meat products.

CTAB – cetyltrimethylammonium bromide.

United Kingdom) and UniProt: the universal protein knowledgebase (The UniProt Consortium, Cambridge, United Kingdom) were used. In case of experimental testing, DNA isolated from 11 organisms (cattle, pig, chicken, duck, turkey, horse, goose, quail, salmon, mackerel and human buccal swab) were analysed under repeatable conditions [26]. Two-fold and four-fold dilution of DNA were used.

Qualitative quadruplex PCR

PCR amplification was conducted in a volume of 15 μl containing 1.5 mmol·l^{-1} $MgCl_2$, 0.2 mmol·l^{-1} dNTP mix (Promega), primer mix, 100 ng template DNA (4 μl) and 0.4 U Platinum DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The common forward primer SIM and reverse primers for beef (B), pig (P), poultry (C) and horse (H) were used (Tab. 2).

Primers SIM, B, P, C and H were mixed in the ratio of 1:0.6:0.6:1.5:1.5 (the ratio 1 means concentration 0.4 μmol·l^{-1}) and used together for the quadruplex PCR. Amplifications were done in Biometra T-Gradient PCR cycler (Whatman Biometra, Göttingen, Germany) using a temperature programme consisting of the initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s, and final polymerization at 72 °C for 5 min. Separation of PCR products was done by electrophoresis in 2.5% or 3% agarose gel (Bio-Rad, Hercules, California, USA) and visualization was done by staining with Midori Green Advance (Elisabeth Pharmacon). For inhibition testing, SYBR Green I intercalation dye (Bio-Rad) was added to mastermix at 0.1 % (v/v). The evaluation was performed according to instructions published regarding verification of analytical methods for GMO testing [26].

Quantitative multiplex qPCR

Primers and probes were complementary to single-copy chromosomally encoded gene sequences. Analyses were carried out using PCR QuantiTect Multiplex NoRox reagent (Qiagen, Hilden, Germany) with 4 μl of DNA in each reaction. Triplex reaction conditions were adopted from Iwobi et al. [22]. Duplex qPCR amplification was conducted in a volume of 25 μl containing QuantiTect Multiplex NoRox reagent (Qiagen), 4 μl template DNA and ChIn_F at 0.05 μmol·l^{-1}, ChIn_R 0.30 μmol·l^{-1}, ChIn_p 0.20 μmol·l^{-1}, My-F and My_R 0.30 μmol·l^{-1} both and My-P 0.20 μmol·l^{-1}. Amplification temperature profile was the same as for triplex qPCR: initial denaturation at 95 °C for 15 min followed by 40 cycles of 30 s at 95 °C and 60 s at 60 °C [22]. The quantitative analyses were performed on ABI 7500 (Applied Biosystems, Foster City, California, USA), the 7500 Software v2.0.6 (Applied Biosystems) being employed for data analysis. Four fluorescence channels were analysed separately.

The procedures for verification of the methodology (amplification efficiency, coefficient of determination, repeatability and trueness) were taken from the dossier on verification of analytical methods for GMO testing [26]. Absolute quantification was carried out by comparing with the standard, e. g. a sample with a known amount of DNA. Single-species DNA, mixtures of DNA and mixtures of homogenized muscle meat were used for verification of quantification. Two calibration curves were constructed for each animal DNA, i. e. one species-specific curve and the second one for reference gene of myostatin, to facilitate relative quantification. Models and samples were analysed in at least duplicates and two independent runs. Amounts of 100 ng and 25 ng of DNA from meat samples were analysed.

Relative quantitative data for one type of meat were calculated by the ratio of copy numbers of haploid genome equivalent (HGE) of this meat towards to HGE of the reference myostatin gene (Eq. 1). The number of HGE in the reaction was calculated from the average published genome sizes, i. e. 1.27 pg for chicken (*Gallus gallus domesticus*); 3.17 pg for pig (*Sus scrofa domesticus*);

3.62 pg for beef cattle (*Bos taurus*) and 3.22 pg for horse (*Equus caballus*) [27].

$$x = \frac{s}{r} \times 100 \quad (1)$$

where x is proportion of animal species DNA expressed in percent, s represents the number of HGE of DNA of one species (beef, pork or chicken) in the samples as calculated from the respective standard curves and r is number of HGE of the endogenous universal myostatin gene (reference DNA).

RESULTS AND DISCUSSION

Multiplex PCR was used for identification of the origin of meat in this study. The procedure was composed of five successive steps (the diagram is shown in Fig. 1). The method consisted of homogenization of the meat or whole meat product, DNA isolation, DNA quality and quantity were estimation, DNA dilution to the required concentration (6.25 ng·μl^{-1} and 25 ng·μl^{-1}) and DNA analysis by PCR. Two possible platforms were proposed and tested, namely, qualitative by endpoint PCR and quantitative by real-time PCR. Quadruplex PCR based on mitochondrial cytochrome b gene amplification was used for qualitative analyses. Triplex and duplex quantitative PCR, based on amplification of single-copy chromosomally encoded gene sequences, were used. Each laboratory may choose which design is more suited for routine sample analysis. Endpoint PCR requires post-PCR analysis which prolongs it and can cause contamination in the laboratory which can be a source of false positive results. From the other point of view the endpoint PCR is low cost compare to qualitative PCR for chemicals and also for necessary laboratory equipment.

DNA isolation

The yield and quality of DNA isolated from pure muscle meat using the CTAB method were higher than 90 ng·μl^{-1} and ratio of absorbances measured at 260 nm and 280 nm (A_{260}/A_{280}) was higher than 1.6 for all tested species. The highest DNA yield (> 180 ng·μl^{-1}) was obtained from chicken meat. Average DNA concentrations obtained from meat products are shown in Tab. 3. An

Tab. 3. Summary of results on analysis of meat products from the market.

Product	c [ng·μl^{-1}]	*RA**	Results of qualitative multiplex PCR				Agreement with declared composition	Results of quantitative PCR [%]			Agreement with declared composition
								Duplex*	Triplex*		
			C/T	H	B	P		C	B	P	
Luncheon meat pork	54 ± 21	1.59	–	–	+	+	Yes	–	3 %	97 %	Yes
Spicy salami	51 ± 14	1.92	–	–	–	+	No	–	–	100 %	No
Pepper sausage	44 ± 12	1.83	–	–	+	+	Yes	–	24 %	76 %	Yes
Sausages	77 ± 11	1.83	–	–	+	+	Yes	–	15 %	85 %	Yes
Veal sausages	109 ±10	1.86	–	–	+	+	Yes	–	18 %	82 %	Yes quality, no quantity
Meatloaf	188 ± 7	1.79	+	–	–	+	Yes	83 %	–	17 %	Yes quality, no quantity
Small sausages I	102 ± 11	1.82	–	–	+	+	Yes	–	6 %	94 %	Yes
Salami RIO EBRO	113 ± 18	1.82	–	–	+	+	Yes	–	13 %	87 %	Yes
Debreciner sausages	146 ± 12	1.83	–	–	+	+	Yes	–	–	100 %	Yes quality, no quantity
Pâté	120 ± 62	1.67	+	–	–	+	Yes	28 %	–	72 %	Yes
Hungarian sausage	219 ± 63	1.80	–	–	+	+	Yes	–	2 %	98 %	Yes
Small sausage II	151 ± 22	1.84	–	–	+	+	Yes	–	10 %	90 %	Yes
Vienna sausage	297 ± 1	1.84	+	–	–	+	No	86 %	–	14 %	No
Luncheon meat	177 ± 7	1.75	+	–	–	+	Yes	38 %	–	62 %	Yes quality, no quantity

c – DNA concentration (expressed as mean ± standard deviation, calculated from absorbance measured at 260 nm), *RA* – ratio A_{260}/A_{280} (ratio of absorbances measured at 260 nm and 280 nm representing the purity of isolated DNA, background absorbance at 320 nm was subtracted), C – chicken DNA, C/T – chicken and/or turkey DNA, B – beef DNA, P – pork DNA, H – horse DNA, * – quantified on the basis of haploid genome equivalents, relative to mammal or poultry DNA.
Legend: (+) – product detected, (–) – product not detected.

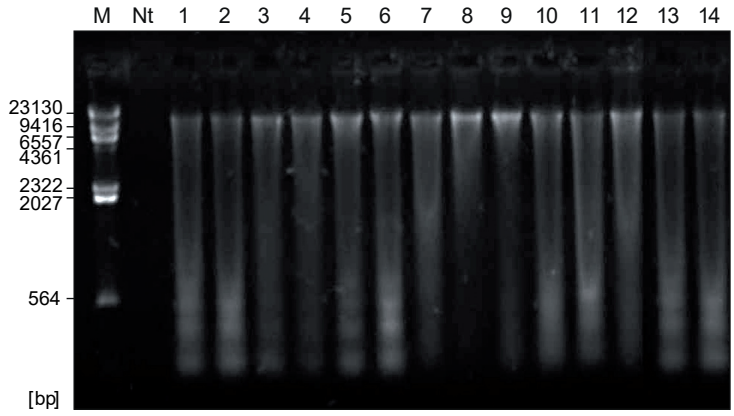

Fig. 2. Electroforetic separation of DNA isolated by CTAB method.

Lanes: M – lambda DNA restricted by Hind III; Nt – no template control; 1, 2 – chicken; 3, 4 – turkey; 5, 6 – duck; 7, 8 – horse; 9, 10 – pork; 11, 12 – beef; 13, 14 – luncheon meat.

average DNA concentration of 35 ng·μl^{-1} was obtained from human buccal swab.

An example of an electrophoretogram of DNA isolated from meat and from commercial meat products is depicted in Fig. 2. This figure shows that isolated DNA was present in sufficient amount and suitable integrity for the following PCR analyses. A phenomenon known as DNA degradation or the splitting into shorter fragments is caused by the influence of higher temperature, lower pH, nucleases, mechanical damage or chemicals [26], which are also used for meat products manufacturing.

In the present study, the effect of temperature was analysed. The results proved that isolated DNA treated by high temperature (including 180 °C) met the qualitative and quantitative requirements for PCR analysis of mitochondrial and genomic DNA.

Qualitative analysis by multiplex PCR of mitochondrial DNA

For qualitative analysis, we relied on the published methodology of Matsunaga et al. [16]. In this study, poultry and human DNA were detected using the published reverse primer marked C. Employing *in silico* analysis with Basic Local Alignment Search Tool (BLAST, NCBI) we proved that primer C published by Matsunaga et al. [16] amplified a 227 bp long segment of cytochrome b from chicken, turkey, duck, quail and human genomes as well. Sometimes, meat may be contaminated by human DNA while being processed in the food industry, which could lead to false-positive results as human DNA would be incorrectly considered as poultry DNA. Therefore, a new reverse primer marked C was used. Its selectivity and specificity were validated by both *in silico* analysis and by experimental analysis in single and multiplex PCR settings at annealing temperatures of 53 °C and 60 °C. The specificity of the primers combination was tested using commercially relevant animal species such as cattle, pig, chicken, duck, turkey, goose, quail, horse, salmon and mackerel. The results were good, as only DNA from the target species was amplified. The detection limits of the quadruplex PCR were 0.01 ng of chicken, 0.01 ng of beef, 0.05 ng of horse and 0.03 ng of pork DNA.

Using mitochondrial DNA for analysis ensures a low detection limit but cannot be used for quantification. Unlike with genomic DNA, which is in most somatic cells in two copies, mitochondrial DNA is present in up to thousands of copies. Moreover, its quantity considerably differs in various tissues. The relatively high degree of mitochondrial DNA mutations, compared to nuclear DNA, leads to accumulation of a number of point mutations, which makes it possible to distinguish closely related species [14, 28].

Meat products are a difficult matrix for DNA analysis with regard to the potential presence of inhibitors as well as DNA degradation due to technological processing including heat treatment, pH fluctuations or pressure changes. The latter may cause difficulties at detection of longer DNA fragments by PCR. Matsunaga et al. [16] could detect a 439 bp fragment of horse DNA in raw meat only, but not in cooked horse meat (autoclaved at 120 °C for 30 min). However, in this study we were able to detect an amplicon of horse meat heated at 120 °C for 30 min. The difference may be due to the use of a different DNA polymerase. The different cooking method may have influenced the result as well, as in this study the meat was dry-

Tab. 4. Summary of quantifications of model samples.

Model	Added DNA [ng]	Mixture composition	Chicken			Beef			Pork			Myostatin		
			THGE	MHGE	[%]	THGE	MHGE	[%]	THGE	MHGE	[%]	THGE	Triplex HGE	Duplex HGE
I	100	B47 : P53	0	N	N	27624	27866 ± 4063	48	31545	32012 ± 6234	55	59170	58489 ± 2790	N
	25	B47 : P53	0	N	N	6907	7050 ± 533	47	7886	7824 ± 188	52	14793	15154 ± 702	N
	12.5	B47 : P53	0	N	N	3453	3480 ± 185	47	3943	3839 ± 112	52	7396	7423 ± 424	N
	6.25	B47 : P53	0	N	N	1727	1600 ± 158	44	1972	1859 ± 8	51	3698	3651 ± 183	N
II	100	C1 : B50 : P49	296	160 ± 84	0.4*	14810	N	N	14514	N	N	29621	N	34187 ± 1615
	100	C5 : B50 : P45	1515	1434 ± 215	3*	15152	17705 ± 1216	59	13636	16766 ± 595	56	30303	30195 ± 1667	56472 ± 1264
	100	C10 : B50 : P40	3120	2693 ± 27	9	15601	19352 ± 542	60	12480	11233 ± 1195	35	31201	31808 ± 3224	31385 ± 231
	25	C10 : B50 : P40	780	622 ± 61	9	3900	4283 ± 70	69	3120	2325 ± 148	38	7800	6191 ± 137	7210 ± 175
	100	C25 : B50 : P25	8730	6346 ± 276	22	13095	12589 ± 424	47	13095	12387 ± 307	46	34919	26900 ± 1344	29538 ± 13
	25	C25 : B50 : P25	2183	1783 ± 75	91	3274	2960 ± 282	41	3274	3129 ± 77	44	8730	7188 ± 665	6140 ± 1084
	25	C100 : B0 : P0	19685	18463 ± 1988	91	N	N	N	N	N	N	19685	N	20379 ± 2960
III	100	C47 : B16 : P19 : H18	19685	18565 ± 485	43	6906	6823 ± 467	18	7886	7707 ± 1478	20	42242	38618 ± 1067	42856 ± 1227
	25	C47 : B16 : P19 : H18	4921	5557 ± 1092	46	1727	1787 ± 172	18	1972	1887 ± 756	19	10560	10039 ± 1042	11528 ± 2077
	6.25	C47 : B16 : P19 : H18	1230	1150 ± 125	51	432	452 ± 44	22	493	475 ± 43	23	2640	2032 ± 243	2238 ± 136
	1.6	C47 : B16 : P19 : H18	308	192 ± 20	37*	108	112 ± 5	20*	123	131 ± 7	24*	660	547 ± 99	526 ± 101
IV		B1 : P99	N	N	N	nd	3 ± 0.1	0*	nd	10449 ± 473	122	nd	8600 ± 249	N
		B5 : P95	0	N	N	nd	2836 ± 277	6	nd	59785 ± 2385	118	nd	50630 ± 673	N
			0	N	N	nd	860 ± 40	6	nd	15479 ± 109	103	nd	15059 ± 922	N
		B10 : P90	N	N	N	nd	943 ± 60	17	nd	5513 ± 96	102	nd	5410 ± 38	N
		B25 : P75	N	N	N	nd	3234 ± 145	33	nd	6892 ± 15	71	nd	9774 ± 950	N
		B50 : P50	0	0	0	nd	38958 ± 173	58	nd	31488 ± 350	47	nd	66802 ± 2645	N
			0	N	N	nd	16472 ± 785	61	nd	14377 ± 282	53	nd	27027 ± 2232	N
			0	0	0	nd	8329 ± 206	65	nd	7176 ± 416	56	nd	12719 ± 1797	N
			0	0	0	nd	2720 ± 114	65	nd	2203 ± 100	53	nd	4165 ± 49	N
		B75 : P25	N	N	N	nd	51773 ± 95	73	nd	17955 ± 746	25	nd	70831 ± 1749	N
			N	N	N	nd	13618 ± 183	85	nd	4243 ± 127	27	nd	15953 ± 55	N
			N	N	N	nd	5692 ± 199	74	nd	2117 ± 53	28	nd	7651 ± 453	N
		B90 : P10	N	N	N	nd	5919 ± 54	89	nd	801 ± 64	12	nd	6638 ± 39	N
		B95 : P5	N	N	N	nd	50907 ± 1329	95	nd	4152 ± 30	8	nd	53324 ± 2076	N
			N	N	N	nd	14519 ± 2157	92	nd	1222 ± 90	8	nd	15752 ± 1319	N
			N	N	N	nd	5259 ± 346	95	nd	423 ± 27	8	nd	5507 ± 546	N
		B99 : P1	N	N	N	nd	8164 ± 909	98	nd	293 ± 46	4	nd	8351 ± 559	N

Models: I – mixed DNA (cattle, pig), II – different mixtures of DNA (chicken, cattle and pig), III – mixed DNA (chicken, cattle, pig and horse), IV – minced meat mixtures from beef and pork homogenized muscle meat.
C – chicken, B – beef, P – pork, H – horse, THGE – theoretical values of haploid genome equivalent, MHGE – measured values of haploid genome equivalent (expressed as mean ± standard deviation), N – not analysed, nd – not determined, * – amount suitable only for qualitative analysis.

heated, not autoclaved. Similar results were published by Ali et al. [29], where a DNA fragment of 411 bp from a sample of pork autoclaved for 2.5 h was barely detected only in the case of 100% meat, not in meat mixtures. Likewise, Bottero et al. [30] tested the influence of cooking (autoclaving at 121 °C for 15 min) and found that DNA fragments of 376 bp from slightly cooked samples could be amplified. However, the authors preferred shorter DNA fragments of 300 bp to obtain better results of DNA amplification from cooked meat.

Quantitative analyses of chromosomal DNA by multiplex quantitative PCR

A method of quantitative PCR was used to determine the proportion of individual meat species. The sequence of the gene encoding for cyclic-GMP-phosphodiesterase was used as a marker for the detection of beef DNA. Pork DNA was detected targeting the sequence of the gene encoding for beta-actin. Chicken DNA was detected targeting the exon-intron DNA sequence of interleukin-2 (Il-2) gene. Individual fractions were quantified against the myostatin gene, which is a universal sequence commonly found in mammals and poultry. All above mentioned genes are present in single copies in the target organisms. Quantification of beef and pork was accomplished by triplex quantitative PCR according to the protocol published by Iwobi et al. [22], while quantification of chicken meat with duplex quantitative PCR proposed in this study (Tab. 2). At designing the system, we referred to the study of Laube et al. [31]. The published amplicon was 95 bp long and positioned inside the Exon 3 of interleukin-2 gene (accession number NM_204153.1, NCBI). In this study, a different reverse primer was used, the sequence of this primer coming from the crossing of the exon 3 and the intron 3 part of the chicken interleukin-2 gene. This section of the intron sequence was chosen because of its greater variability within closely related animal species, compared to that found in exons. Another reason for choosing an intron was to prevent the primer from binding to mRNA.

The specificity of the primer systems was successfully proven by PCR with DNA extracted from authentic samples of the muscle meat from chicken, duck, turkey, pig, cattle, horse, salmon, mackerel, quail, goose and with DNA obtained from a human buccal swab. The efficiency, repeatability and accuracy of were verified by models of various mixtures of isolated DNA and models of muscle meat mixtures (Tab. 4). Calibration curves for quantification had amplification efficiencies of 90–110 %, corresponding to a slope between –3.1 and –3.6. Coefficients of determination R^2 were ≥ 0.98. Relative repeatability standard deviation was ≤ 25 % [26]. The results show that quantitative PCR could precisely quantify DNA of single animal species, even in a mixture. It allowed for the detection of approximately 30 copies of the haploid pig genome, 26 copies of the haploid beef-cattle genome and ≥ 11 copies of haploid chicken genome in a sample. The DNA concentration suitable for precise quantification ranged from 12.5 ng to 200 ng. The detection limits for the triplex quantitative PCR system ranged from 1 % to 5 % of the component portion in the mixture.

The influence of heat treatment of meat samples on PCR

The PCR systems were tested for performance with both raw and heat-treated beef, pork, horse and chicken meat. DNA was extracted from all the aliquots (raw mixtures and mixtures heat-treated at temperatures of 70 °C, 100 °C, 120 °C and 180 °C for 30 min in a hot-air oven). The calibration curve at 1:3 dilution of DNA solution with an initial concentration point of 25 $ng \cdot \mu l^{-1}$ was constructed. The analysis of the obtained calibration curves proved that it was possible to quantify heat-treated samples containing partially degraded DNA. Coefficient of determination R^2 and effectiveness were not significantly different for DNA isolated from raw meat and DNA isolated from heat-treated meat (Tab. 5).

Analysis of commercial samples

The proposed systems were used to analyse 14 meat products bought in the market (Czech Republic). The obtained results were compared with to the data declared on the packages. In

Tab. 5. Statistical parameters of the calibration curves for PCR analysis of DNA isolated from raw and heat-treated meat.

	Raw	Heat-treatment temperature			
		70 °C	100 °C	120 °C	180 °C
Slope	–3.42	–3.45	–3.40	–3.41	–3.25
Coefficient of determination R^2	1.0	1.0	1.0	1.0	1.0
Efficiency [%]	96.0	95.0	96.9	96.6	103.0

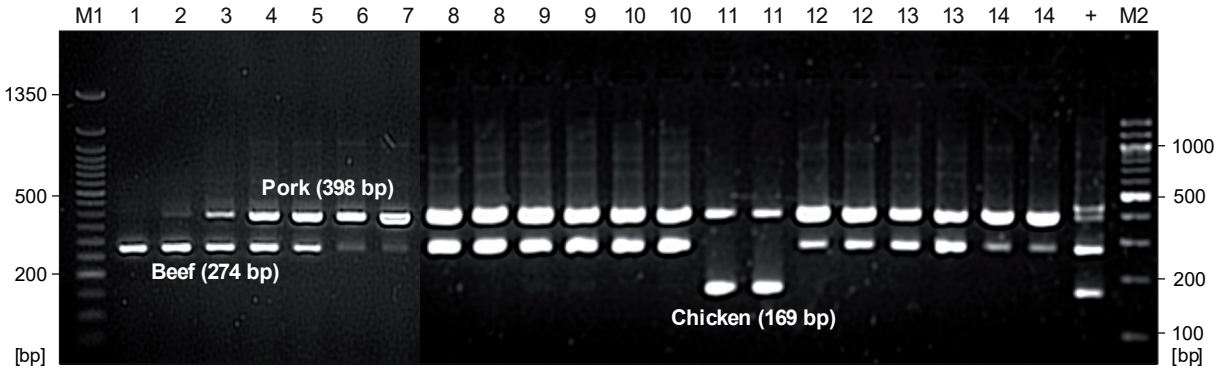

Fig. 3. Amplification by quadruplex PCR.

Lanes: M1 – standard ($n \times 50$ bp), 1 – mixture P1 : B99 (where P stands for pork and B for beef), 2 – mixture P5 : B95, 3 – mixture P25 : B75, 4 – mixture P50 : B50, 5 – mixture P75 : B25, 6 – mixture P95 : B5, 7 – mixture P99 : B1, 8 – pepper sausage, 9 – sausage, 10 – veal sausage, 11 – meatloaf, 12 – small sausage I, 13 – salami RIO EBRO, 14 – Debreciner sausage, (+) – mixture of chicken, cattle, pig and horse DNA, M2 – standard ($n \times 100$ bp).

ten cases, the percentages of different kinds of meat contained in the products were stated on the packaging. The information about the total amount of meat (a mixture of beef and pork) was written on four products (Tab. 1).

The amount of DNA in the range of 44–297 ng·μl^{-1} was isolated (Tab. 3). DNA at a concentration of ≥ 100 ng·μl^{-1} was isolated from a pâté. Only 50 ng·μl^{-1} of DNA could be isolated from luncheon meat pork. In addition, the luncheon meat pork DNA preparation was contaminated with proteins, the ratio A_{260}/A_{280} being 1.6. Liver is used for the preparation of pâté and it contains a greater amount of DNA than muscles, so higher DNA yield could be probably achieved. On the contrary, fatty products (luncheon meat or meat loaf) or products with a large content of skin contain less DNA and, therefore, lower yields of DNA could be isolated from such samples.

Quadruplex PCR was used for qualitative analysis, triplex quantitative PCR was used for analysis of beef and pork DNA, and duplex quantitative PCR was used for analysis of chicken DNA. The results of multiplex PCR analysis are shown in Fig. 3 and summarized and evaluated in Tab. 3. The qualitative results, which show the presence or absence of the tested DNA of both targets (mitochondrial and genomic DNA) were identical for 13 meat products except for the Debreciner sausage. Due to its high copy number, one important advantage of amplifying mitochondrial DNA is its lower detection limit. This might explain differences in the results of the analysis of the Debreciner sausage. Although mitochondrial DNA is not suitable for quantitative analysis, it is well suited for the screening of products on the market.

The information declared by the producer corresponded to the results of qualitative and quantitative PCR in 8 out of 14 samples: luncheon meat pork, pepper sausage, sausages, small sausages I, salami RIO EBRO, pâté, Hungarian sausage and small sausage II. While comparing declared meat contents, a higher content of pork meat and a lower content of beef meat (10 %) were detected in small sausage II. This might have been caused by the addition of pork lard and skin to the sample by the producer.

In two of the tested samples, the declared composition was found to be untrue. In the spicy salami, only pork was detected but no beef, while the producer had claimed it contained 31 % of it. A similar result was obtained in the case of the Vienna sausage, in which only chicken and pork were detected, while beef was not detected by either qualitative or quantitative analysis. The determined content of chicken and pork meat were comparable with the label although a slightly higher value of chicken component was found. This might have been caused by the presence of various tissues from mechanical separation of the meat. In four samples (veal sausage, meatloaf, Debreciner sausage and luncheon meat), the results of qualitative multiplex PCR analysis confirmed the proclaimed composition. However, quantitative analysis revealed differences in the declared meat contents in the products. The stated content of the meatloaf did not correspond with contents of individual components. Analysis showed a significantly higher content of chicken meat and lower content of pork. However, a lot of fat might have been included in the pork meat, as there was an "acceptable tolerance of 30 %" stated on package labeling [22]. That is one reason why precise results could be slightly lower than expected, if

a portion of pork meat was replaced with chicken meat. On the other hand, the luncheon meat showed a higher amount of pork than chicken DNA. In veal sausages, only 18 % of beef meat content was identified quantitatively, while the declared value was 54 %. In the Debreciner sausage, beef DNA was positively detected by qualitative PCR but not determined by quantitative PCR. As the label of this product did not disclose any information on the percentage of components, this product could not be evaluated as adulterated.

Various animal body parts are used for preparation of meat products, which presents challenges in the quantification of meat content in meat products. The cell number per weight unit and the amount of extracted DNA depend on the type of tissue [32]. Generally, more DNA can be isolated from internal organs than from muscles. The presence of kidneys, hearts or livers in a meat product can cause overestimation of the quantity ratio of any particular animal species [31]. Problems can then arise from the lack of knowledge about the accurate composition and degree of processing of a product. This is the reason why methods based on DNA analysis or protein analyses do not provide precise results, which would directly correspond with the amount of meat (w/w) in a given product. Quantification of individual components in meat products focusing on the ratio of individual genomes (genome/genome) provides very precise results [14]. Reference material containing different meat fractions (ratios) is needed. Currently, certified reference material with the composition of different meats is not available, unlike in the case of GMO detection.

Our results on a level of incorrect information on labels of meat products are in line with previous results of studies in other countries. Ballin [14] cited statistical data in which approximately 20 % of meat products in USA, 22 % in Turkey, 15 % in Switzerland and 8 % in the United Kingdom were poorly labelled. An analysis of 139 samples of processed meat products from retail outlets and butcher shops in South Africa was performed [33], the results showing that of the 139 samples, 95 (68 %) contained species that were not shown on the label. The presence of traditional species, such as donkey, goat or buffalo in several products was found. In Turkey, beef DNA was not detected in samples of beef sausage (5 % of beef was declared) and chicken and turkey DNA was found in meat balls declared as 100% beef [34, 35]. PCR analysis of 72 packaged meat products from the Italian market showed that 41 products (57 %) were incorrectly labelled [36]. Ayaz et al. [37] found that of 100 analysed raw and heat-treated products, 22 % contained undeclared species (e. g. poultry meat). In our pilot study, we found contradicting information between the product label and the qualitative results in two out of fourteen products (14 %). The DNA ratios of three animal species were quantified (chicken, pork and beef), and a contradiction was found between the proportion declared on the label and the DNA content in four more of the analysed samples.

CONCLUSIONS

Meat authentication in food using PCR is a quite simple, cost-effective and robust method. This analysis enables both quantitative and qualitative measurements to be accomplished quickly, even with a scarcity of the original DNA. Qualitative endpoint PCR allows for the screening of meat and meat products. Quantitative PCR is somewhat more costly compared to endpoint PCR, but it allows for simple and more precise evaluation of the analysis. Multiplex PCR saves both time and money compared to single target analyses. In this study, multiplex analysis of mitochondrial and genomic DNA was performed, which is an original approach. Thanks to short amplicons, quantitative multiplex PCR techniques make it possible to carry out reliable tests of heat-treated meat products for the presence of chicken, beef and pork DNA. Moreover, quantitative PCR methods contain a cross-check amplification of a myostatin gene fragment of mammals and birds in their protocol. This cross-check reaction helps to verify amplification and/or it may be used to quantify animal DNA. The results of the analysis of the meat products from the market in Czech Republic showed a level of adulteration. The proposed PCR-based methodology proved to be effective and quite simple for detection of meat adulteration.

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