

Detection of meat adulteration in veal sausages using a multiplex PCR technique

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Abstract: *Previous methods used to detect veal contamination by lower value meat lacked easiness and accuracy. This study describes a simple procedure for detecting origin of meat in processed meat products. A rapid (5h) protocol based on multiplex PCR was developed to detect undeclared chicken meat (a specific mitochondrial DNA sequence) in Polish veal sausages. Simultaneously a PCR assay was successfully optimized for amplification of 274-bp DNA fragment extracted from meat products using designed species-specific primer pairs for the detection of veal meat. This procedure also enabled differentiation of bovine or/and chicken meat from their less expensive porcine substitute.*

Keywords: *food adulterations, meat, multiplex PCR.*

Introduction

Food product which misleads consumers by undeclared substitution of one of its constituents is considered to be adulterated. Food adulteration is usually intentional in order to lower the cost of production and to obtain the higher profit [1, 2, 3]. Currently, scientific research institutes and food inspection services are facing the necessity of determining food constituents for health and economic reasons [4, 5, 6]. It is mainly due to the increasing phenomenon of allergies as well as more frequently reported cases of replacing more expensive food ingredients with their cheaper substitutes [7, 8, 9, 10].

In many cases most commonly used detection methods based on physicochemical and immunological analyses do not entirely fulfill their aims because, they neither guarantee reproducibility nor are very reliable [11, 12] and are as diverse as the authentication problems [13, 14]. In comparison with protein tests, qualitative and quantitative DNA-based methods (like PCR techniques) have proved to be more reliable because of the stability of DNA structure under the food processing conditions associated with high temperatures, high pressures, chemical treatments, sterilizing, smoking, salting [2, 15, 16, 17].

The PCR technique has been utilized for animal species identification. Its well-recognized amplification potential means that the technique can be exceptionally sensitive and offer a new scope for the analysis of samples, which because of their low levels of target DNA, could not be tested by using other methods [3, 18, 19].

Experimental

In this study, the performance of multiplex PCR-based method with the use of species-specific primers to screen for undeclared chicken meat in veal sausages was tested. The objective was to develop a protocol that could successfully be used in routine control assays to detect undesirable meat species in comminuted meat products.

In order to assess and control contaminations developed during molecular analyses, the following control samples were introduced (acc. to ISO 24276:2006 and guidelines of the European Union Reference Laboratory for animal proteins in feedingstuffs, EURL-AP) [20].

Materials

Samples of meat products (veal sausages, n=30) were obtained from local supermarkets and stores in West Pomeranian Province, Poland and stored up to max. 2 days at 4°C until analysis (Table 1).

Table 1. Declared ingredients and obtained results for veal sausages

Sample ID of veal sausages	Declared ingredients	Results		
		veal	chicken	pig
01	veal, pig	+	+	+
02	veal, pig	+	-	+
03	veal, pig	+	-	+
04	veal, pig	+	+	+
05	veal, pig	+	-	+
06	veal, pig	+	-	+
07	veal, pig	+	+	+
08	veal, beef & pig	+	-	+
09	veal, beef & pig	+	-	+
10	veal, beef & pig	+	-	+
11	veal, pig	+	-	+
12	veal, pig	+	-	+
13	veal, pig	+	-	+
14	veal, pig	-	+	+
15	veal, pig	+	-	+
16	veal, pig	-	+	+
17	veal, beef & pig	+	-	+
18	veal, pig	+	-	+
19	veal, pig	+	-	+

Table 1 (continued)

20	veal, pig	+	-	+
21	veal, beef & pig	+	-	+
22	veal, pig	+	-	+
23	veal, pig	+	-	+
24	veal, pig	+	-	+
25	veal, pig	+	-	+
26	veal, pig	+	+	+
27	veal, beef & pig	+	-	+
28	veal, beef & pig	+	-	+
29	veal, beef & pig	+	-	+
30	veal, beef & pig	+	-	+

Methods

DNA isolation. Total genomic DNA from meat products was extracted using a modified version of Genomic Mini AX Food extraction kit (A&A Biotechnology, Poland). Tissue (150 mg) was ground in a sterile mortar and transferred into a 2-mL tube. Samples were treated with 1,5 mL of Lysis solution and 20 μ L of Protease to initiate the cell lysis. The next step was the incubation for 50 min at 53°C followed by intensive shaking for 15 s (BioVortex V1 minishaker, Biosan). After cooling down for 5 min at a room temperature, the tubes were centrifuged at 13000 rpm/min. The surface fraction was removed and placed in the an anion exchange membrane. DNA was eluted from the column with high ionic strength solution and collected in a new tube. Isopropanol (800 μ L) was added and mixed carefully by inverting the tube 10-20 times. Samples were centrifuged for 10 min at 10000 rpm/min. and supernatant was carefully removed before washing the pellet with 0.5 mL of 70% ethanol. Then, samples were centrifuged again for 5 min at 10000 rpm/min. Supernatant was carefully removed and pellets were air-dried for ca. 10 min by placing the tubes upside-down on the tissue paper. After drying DNA pellet was dissolved in the TE buffer.

The nucleic acid concentration was measured by a spectrophotometric analysis (Biophotomer, Eppendorf, Germany) and extracted DNA was used as a matrix for PCR. The quantity and purity of extracted DNA present and 260/280 ratio for each sample was more than 200 ng/ μ L and a 260/280 ratio > 1.75. DNA was stored either at 4°C (short-term) or at -32°C (long-term).

Primers and PCR conditions. Species-specific primers designed for the detection of bovine (veal), pig and chicken DNA (Table 2) and targeting species-specific sequences of mitochondrial cytochrome b genes were used [21].

Table 2. Species-specific primers sequences used in multiplex PCR

Species	Primers (5'-3')	Target	Size (bp)
common forward primer [SIM]	gACCTCCCAgCTCCATCAAACATCTCAT CTTgATgAAA		
<i>Bos Taurus</i> [cattle]	CTAgAAAAGTgTAAgACCCgTAATATAAg		274
<i>Gallus gallus</i> [chicken]	AAGATACAgATgAAgAAgAATgAggCg	cytb mt DNA	227
<i>Sus scrofa</i> [pig]	CTATgAATgCTgTggCTATTgTCgCA		398

The amplification of meat DNA was performed in a final volume of 25 μ L in a PCR thin-walled tube (Eppendorf AG, Germany) containing 250 ng of template DNA, 1 unit of Taq polymerase (A&A Biotechnology, Poland), 2.5 units of polymerase Taq buffer (100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM TrisHCl pH 8,5, 20 mM MgSO₄, 1% triton X-100), 25 mM MgCl₂ (Quiagen, Germany), 2,5 mM dNTP (Fermentas, Latvia) and primers: SIM – 20 pM, chicken – 60 pM, cattle – 12 pM and pig – 12 pM.

The amplification was performed in Mastercycler Gradient (Eppendorf AG, Germany) in the following temperature conditions - after an initial denaturation step at 94°C for 5 min, 35 cycles were programmed as follows: denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s and primer extension at 72°C for 30 s. The last extension step was 5 min longer. An electrophoresis of a 10 μ L portion of amplified DNA was carried out for 120 min at 45V in 2% agarose gel (Prona Agarose Plus, Belgium) containing ethidium bromide (Bio-Rad, USA) (1 μ L/mL) in TBE (AppliChem, Germany). After DNA migration, the results were visualized under UV light (GelDoc 2000 Bio-Rad, USA).

Results and Discussion

In a preliminary phase of the investigation, simplex PCR was performed on DNA extracted from raw meat to verify the specificity of the primers. Primers used generated specific fragments of 227bp for chicken, 274bp for beef (veal) and 398 bp for pig (Figure 1). No cross-reaction was reported. Each test was repeated three times and gave reproducible results.

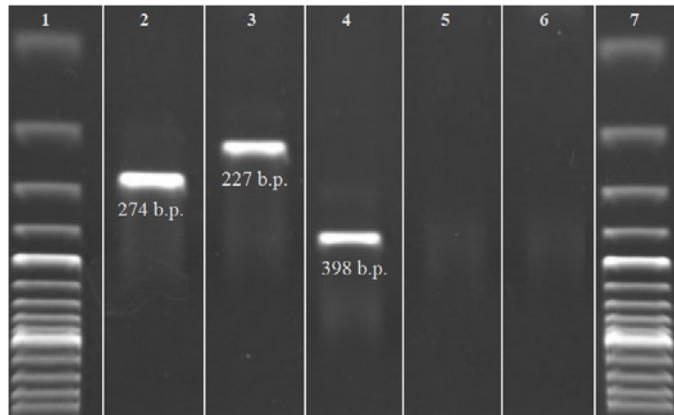


Figure 1. Species-specific primers tested: 1, 7 – molecular mass marker, lane 2 – veal meat, lane 3 – chicken meat; lane 4 – pig meat, lane 5 – negative control (horse meat), lane 6 – NTC, no template control. (2% agarose gel, ethidium bromide-stained)

The applicability of the multiplex PCR assays combining both primers (cattle + chicken) and common forward primer (SIM) to analysis of processed meat products has also been demonstrated.

Six from thirty examined sausages contained undeclared chicken meat (Table 1). Despite the declaration of producers and/or sellers, two of the examined sausages did not contain veal (or beef) meat at all (Figure 2). All examined products contained declared pork meat.

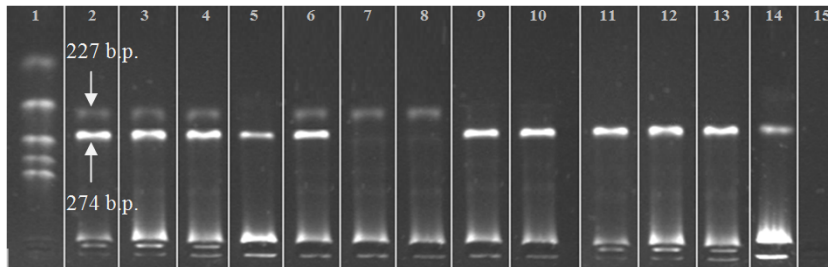


Figure 2. Veal product tested: lane 1 – the marker of molecular mass, 2-14 veal products, 15 – negative control (horse meat) (2% agarose gel, ethidium bromide-stained)

The complexity of technological process of sausage production and limitations of methods used for detecting food adulteration in highly processed food enable some producers/retailers to engage in illegal practices like substitution or removal of valuable/expense ingredients.

Therefore, implementation of reliable and rapid methods of identification of various meat types in processed meat products is crucial for food quality control due to various legal, economic, religious, and health-related issues.

Since DNA is a temperature-stable structure and does not degrade even during fermentation, maturing, salting or smoking of food products [2, 22, 23] the PCR technique has been successfully applied for animal/meat species identification, for example pork, horse and donkey meat in cooked sausages and meat patties [17, 24], poultry in commercial sausages and minced meat products [1] or hare meat in mixtures containing pork meat [25].

In this study, the species-specific single-step PCR protocol was developed for the identification of beef, chicken and porcine species in the sausages based on primers proposed by Matsunaga *et al.* [21]. The multiplex PCR assay was designed based on the sequence of mitochondrial cytochrome b gene present in DNA of all tested meat species. Mitochondrial gene sequences are often used in identification of animal products because mtDNA comprises up to 2% of total DNA in animal cells, is highly conserved, maternally inherited and contains species-specific heterogenic loci [26]. Also, if compared with nuclear DNA, mtDNA is not tightly bonded proteins and this significantly facilitates its extraction [12, 27]. Primers used in our study generated specific fragments for chicken, and bovine meat, respectively, and amplification was not affected by spice addition or technological process. Similar results were found in cooked meat and sausages [21, 28]. López-Andreo *et al.* [24] stated that, investigated species can be detected in cooked sausages without any detrimental influence of composition and processing conditions. Haunshi *et al.* [15] showed, that the used DNA markers were useful for identifying species origin of cooked and autoclaved meat samples. Also Sawicki [2] who analysed minced, frozen, cooked, smoked and sterilised meat products, stated that the applied PCR protocol determined the species identity of raw materials in 100% of cases of processed products.

Two factors: quality and purity of isolated DNA strongly determine the result of PCR amplification. Processed food may contain PCR inhibitors, like sodium acetate, sodium chloride, haemoglobin, and heparin. To eliminate or reduce inhibitors, a proper method of nucleic acid isolation must be chosen [2].

It has been reported that the extraction method based on the binding of DNA to a silica matrix in the presence of chaotropic agents is more efficient and removes the PCR reaction inhibitors [13].

Thus, in our study, a commercial DNA isolation kit based on silica columns was preferred to minimize potential human errors and provide required reproducibility.

Our protocol proved not only to be rapid (approx. 5 h from receiving the sample to delivering final results) but also sensitive. It enabled detection of 0,05 ng of template DNA of each species when assessed using dilutions of DNA in TE buffer. With the species-specific multiplex PCR assays, we reached

a detection limit of less than 0,1% of substituted meat in the sausage samples for beef, chicken and porcine DNA [22, 24]. This confirms results of our previous studies in which we identified bovine and porcine DNA (0,1% of substituted meat in final product) in temperature-treated samples (100°C/30 min and 121°C/15 min) [19].

Using the same set of primers Matsunaga *et al.* [21] detected 0.25 ng DNA in 6 different species using multiplex PCR. Calvo *et al.* [26] utilised PCR reaction to evaluate semi-quantitatively – based on the number of PCR cycles – content of particular meat species added to the meat product. The upper limit of pork detection in beef products was at 0,005% level and 1% in duck liver pâté. Lopez-Andreo *et al.* [24] and Hird *et al.* [22] reported that thermal processing did not limit a detection scope. The limit was established at 1% for pork, lamb, and goat meat in final product when treated at 65°C for 30 min and 121°C for 20 min.

It should be taken into account, that this sensitivity is, however, potentially problematic since a low level of adventitious contamination is often permitted by food labelling legislation [9, 25, 29]. A common argument against the use of PCR based techniques is that they are too sensitive and that minute traces of material would/can produce a positive result.

Conclusion

We developed a fast (5h) and reproducible protocol for the identification of veal meat in processed meat products based on multiplex PCR amplification of the bovine mtDNA without cross-reaction with other animal species DNA with detection limits less than 0,1% of substituted meat.

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