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Full Length Research Paper

Qualitative analysis of meat and meat products by multiplex polymerase chain reaction (PCR) technique

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Multiplex polymerase chain reaction (M-PCR) assay was applied to processed and raw meats for the identification of the most used species in foodstuffs such as, ruminant, poultry, fish and pork materials. Specific-species primers, designed according to the conservative regions of 16S rRNA, were used after alignment of the available sequences in the GenBank database. The primers generated specific DNA fragments of 183, 224, 290 and 374 bp length for poultry, fish, pork and ruminant, respectively. The optimized M-PCR assay was applied to 93 commercial meat products and it showed the presence of poultry meat in red meat analyzed, although, it was not indicated on the label.

Key words: Multiplex polymerase chain reaction (M-PCR), meat products, food, salami, sausage.

INTRODUCTION

Meat and meat products include nutrients that humans require for growth, physiological functions and body health. The consumption and production of food in convenient conditions (hygienic production, correctly labeled food), is a vital phenomenon for human life. It is generally accepted in the food sector that a diet meat and meat products should provide healthy nourishment for consumers.

There has been an increase in the consumption of a number of quality products and a position change in this respect. Currently, consumers want to buy high quality products, which have a good-label. However, false or accidental mislabeling still exists and may not be detected, resulting in poor quality products. People with an allergy against special meat and Its products do not demand combined meat products having allergen materials, as their health will be endangered.

In most countries, food manufacturers choose to use some products instead of others, such as lard as a substitute ingredient for oil or chicken meat as a substitute ingredient for red meat, because they are cheaper and easily available. Biological complications and health risks may be associated with a daily intake of these products. Hence, it is an important task for food control laboratories to be able to carry out species differentiation of the raw materials to be used for industrial food preparation and the detection of animal species in food products (Aida et al., 2005).

The European Union (EU) has implemented a set of very strict procedures for the labeling of food. Throughout the whole legit procedure, the EU ensures the European consumers' rights. Thus, analytical methods for the molecular determination of food are essential in order to verify suitable labeling (Pinto et al., 2005). In Turkey, according to the one hundred and forty-seventh article of the food law, the species' names of meats used to prepare the meat products have to be presented on the label of the product. Moreover, selling other meat species with false labels to get more profit is held as imitation and prohibition according to the foodstuff laws.

Many analytical methods that rely on protein analysis have been developed for identification of species. These

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Abbreviations: ELISA, Enzyme-linked immunosorbent assay; **IEF,** isoelectric focusing; **PCR,** polymerase chain reaction; **RAPD,** random amplified polymorphic DNA; **RU,** ruminant; **PO,** poultry; **FS,** fish; **PR,** pork; **EDTA,** ethylenediaminetetraacetic acid; **BLAST,** Basic Local Alignment Search Tool; **NCBI,** National Center for Biotechnology Information.

methods include, liquid chromatography (Meyer and Candrian, 1996), electrophoresis techniques (Kim and Shelef, 1986), immunological methods such as the enzyme-linked immunosorbent assay (ELISA) test (Jones and Patterson, 1985; Hsieh et al., 1998) and isoelectric focusing (IEF) (Pinto et al., 2005). However, these methods differ greatly in sensitivity and specificity. Also, some of the methods are labor-intensive and they require expertise and sophistication. Another determinant limiting the use of some advanced methods is the food processing factor. For example, identification of the origin of meats by ELISA can fail due to denaturation of protein in high temperature. Hence, many researchers have emphasized that, there is a need for simpler, more accurate and rapid techniques for the determination of species of meat, especially in cooked-meat products.

The dot-blot technique was the first genetic approach for the determination of species' identity (Ebbehoj and Thomsen, 1991; Wintero et al., 1991). At present, polymerase chain reaction (PCR) a method for amplification of DNA in an artificial environment has been successfully used for species identification of animals, plants and bacteria (Arslan et al., 2006; Ce´spedes et al., 1999). Some of the PCR approaches used for the determination of the identity of DNA are random amplified polymorphic DNA (RAPD)-PCR (Lee and Chang, 1994) and analysis of different PCR fragments (Meyer et al., 1995; Matssunaga et al., 1999). DNA hybridization methods are complicated and generally inadequate, but PCR easily amplifies the target regions of the DNA template in a much shorter time. PCR is suitable for meat identification and is commonly used. Matssunaga et al. (1999) developed a multiplex PCR method for the identification of six types of meat. PCR has been applied for the detection of bovine tissue in animal feed, because of mad cow disease (Wang et al., 2000; Krcmar and Rencova, 2001). Lahiff et al. (2001) developed a PCR to identify ovine, porcine and poultry DNA in feedstuff. Myers et al. (1995) identified different species in feedstuff by using universal primers coupled with restriction endonucleases. Several workers have developed PCR methods to control the suitability, with labels of meat products. For example, Pinto et al. (2005) optimized a duplex PCR method in order to identify pork meat in horse meat fresh sausages obtained from Italian retail sources. Multiplex PCR assay was optimized by Ghovvati et al. (2009) for fraud identification in industrial meat products by using a three primer set. Bai et al. (2009) developed a novel common primer multiplex PCR method for the simultaneous detection of meat species.

Together with the classic PCR techniques explained earlier, currently, real-time PCR has been used to identify meat and meat products (Andreoa et al., 2005; Chisholm et al., 2005; Miguel et al., 2005; Chun Lai Zhang et al., 2006; Violeta Fajardo et al., 2007; Kesmen et al., 2009). However, in spite of the fact that real-time PCR techniques have more advantages than the classical PCR method, most molecular research laboratories do not have this system, as real-time PCR is relatively expensive and requires experienced hands to operate. On account of this, we need to develop and optimize the classic M-PCR, for the simultaneous and rapid detection of animal species, at least to identify the contamination in complex meat products.

M-PCR is a variant of PCR in which two or more DNA loci are simultaneously amplified in the same reaction. Since its first description in 1988, this method has been successfully applied in many areas of DNA testing (Henegariu et al., 1997). The use of multiple, unique primer sets within a single PCR mixture is to produce amplicons of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that would otherwise require the use of reagents which require several times and a longer time to perform. Annealing temperatures for each of the primer sets must be optimized for them to work accurately within a single reaction and the amplicon sizes, that is, their base pair length, should be different to form distinct bands when visualized on gel electrophoresis.

Multiplex PCR has some advantages which include: (1) Internal control: potential problems in PCR include false negative results due to reaction failure or false positive results due to contamination. False negatives are often revealed in multiplex amplification because each amplicon provides an internal control for the other amplified fragments; (2) indication of DNA template quality: the quality of the template may be determined more effectively in the multiplex than in a single locus PCR. A degraded DNA template gives weaker signals for long bands than for short ones. A loss in amplification efficiency due to PCR inhibitors in the DNA template samples can be indicated by reduced amplification of an abundant control sequence, in addition to the amplification of rarer target sequences in an otherwise standardized reaction (Van der Vliet et al., 1993); (3) indication of template DNA quantity: the exponential amplification and internal standards of multiplex PCR can be used to assess the amount of a particular template in a sample (Edwards and Gibbs, 1994); (4) PCR economical efficiency: the expense of PCR reagents and preparation time is less in M-PCR than in systems where several tubes of uniplex PCRs are used. A multiplex reaction is ideal for conserving the expensive polymerase (Chamberlain et al., 1988); (5) rapid method: M-PCR is a rapid technique because many meat products can be analyzed in the same reaction tube. For example, 100 meat samples can be analyzed in 25 reaction tubes at the same time by using four primer sets belonging to ruminant, poultry, pork and fish.

In this study, we focused on the determination of DNA in different processed and unprocessed meat samples.

The aim of the present study is to optimize and develop an M-PCR technique for detection of ruminant, poultry, pork and fish materials in some industrial meat products, such as, salami, sausage, meat ball, mince and raw meat.

MATERIALS AND METHODS

Sample collection

Meat samples were collected from shops located in different parts of Istanbul. Fifty-seven of 93 were processed and mixed products; 30 and 31 among them were labeled and unlabeled samples, respectively. There was no information about the ratio of the meat species used, on the labels of 19 processed and mixed meat samples. At that time, there were 35 raw samples (mince, etc.) included in the research. Four of the 35 samples were standards that were cut directly from the body of the animals, ruminant (RU), poultry (PO), fish (FS) and pork (PR). Although, the origin of one of the 35 samples was unknown, the origin of the rest was known, as they were obtained from ordinary local shops and supermarkets. Therefore, the collected 93 meat samples were categorized (Table 1).

Preparation of meat samples

Meat samples were collected from different parts of Istanbul between 2007 and 2008, with clean unused plastic boxes to prevent cross contamination. As soon as the samples were obtained, they were directly transported to the Molecular Biology and Genetic Research Laboratory of the Fatih University, at the Buyukcekmece Campus, Istanbul. 20 g of each sample was homogenized by sterile mixers in test tubes, manually. Exactly 25 mg of the sample was placed in a 1.5 ml microcentrifuge tube.

DNA isolation

DNA was extracted from 25 mg of meat samples using DNeasy® tissue kit (Qiagen, Hilden, Germany) as per the Manufacturee's instructions. One hundred and eighty microliters of ATL buffer and 20 µl proteinase K were added and vortexed. The mixture was incubated at 56°C in a water bath, to disperse the sample, until the tissue was completely lysed. The mixture was vortexed for 15 s; 200 µl of AL buffer was added to the sample and vortexed thoroughly. 200 µl of ethanol (96 %) was added to the mixture and it was vortexed to yield a homogenous solution. The homogenous solution was transferred into the DNeasy® mini column in a 2 ml collection tube. The homogenous solution was centrifuged at 8000 rpm for 1 min. The flow-through and collection tubes were discarded and the DNeasy® mini column was put in a new 2 ml collection tube. 500 µl of AW1 buffer was added and spun at 8000 rpm for 1 min. The flow-through and collection tubes were discarded and the DNeasy® mini column was placed in another 2 ml collection tube. 500 µl of AW2 buffer was added and centrifuged at 14,000 rpm for 3 min to dry the DNeasy membrane and then, the flow-through and collection tube were removed. The DNeasy® mini column was placed in a clean 1.5 ml micro centrifuge tube. 200 µl of AE buffer was transferred directly onto the DNeasy® membrane and incubated at room temperature at 1 min followed by spinning at 8000 rpm for 1 min to elute it. Elution was repeated to increase the final DNA concentration. The concentration of DNA was measured by spectrophotometer (UNICAM UV-VISIBLE, VISION SOFTWARE V3.41) the DNA solutions were stored at -20°C.

DNA quantification

The DNA concentration was accounted by the following formula:

DNA concentration = OD_{260} x extinction coefficient (50 µg/ml) x dilution factor

A spectrophotometer device was used to determine the concentration of DNA in the solution. The samples were exposed to ultraviolet light at 260 and 280 nm. A 260:280 ratio was determined as the qualification of nucleic acids.

Primer design and production

We only designed ruminant primers (16S rRNA) using the primer 3 (v. 0.4.0), primer design software (http://fokker.wi.mit.edu/primer3/input.htm) accessed on the internet. Four sets of primers that were used in this study, for multiplex PCR amplification, are listed in Table 2. Three of the species-specific primers (12S rRNA, tRNA Val and 16S rRNA) were published by Dalmasso et al. (2004). The specificity of all the primers (Table 1) were checked by using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Primers were synthesized by Iontek, Istanbul, Turkey.

Simplex PCR

Polymerase chain reaction amplification was performed in a final volume of 25 µl, containing 10 x Taq buffer + $(NH_4)2SO_4$, 1 unit of platinum Taq DNA polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2 mM MgCl2, 0.1 mM of each primer, and 60 to 80 ng/µl of DNA template. Amplification was performed in a Techne thermocycler, with the following cycling conditions: After an initial heat denaturation step at 94°C for 10 min, 35 cycles were programmed as follows: at 94°C for 30 s, annealing at 60°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min.

Multiplex PCR

For the simultaneous detection of each species, a multiplex PCR was developed using each of the primer sets previously designed for simplex PCR. As for the simplex PCR, amplification was performed in a final volume of 25 µl containing 10 x Taq buffer + $(NH_4)2SO_4$, 1.5 units of platinum Taq DNA polymerase (Iontek, Turkey), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Iontek, Turkey), 2 mM $MgCl_2$, 20, 20, 12.5 and 10 pmol of ruminant, pork, fish and poultry primers, respectively and 60 to 80 ng/µl of DNA template. Thermal cycling was programmed following the same procedure used for simplex PCR.

Amplimers were resolved by electrophoresis on 3% agarose gel (MERCK) run in Tris boric ethylenediaminetetraacetic acid (EDTA) buffer for 50 min, at 110 V and monitored under UV light (Bio-Rad GelDoc 2000).

RESULTS

DNA isolation

The results indicated that isolated DNA was adequate for PCR amplification. Some properties of the extracted DNA

Table 1. Grouping of meat samples according to the criteria given herewith.

Reference(standard) species	No. (#)	Percentage (%)
RU	1	1.1
PO	1	1.1
FS	1	1.1
PR	1	1.1
Categorization of labeled products according to their origin		
RU	42	45.2
PO	10	10.8
FS	5	5.4
PR	1	1.1
UK	22	23.7
Categorized according to the type of process		
PM	57	61.3
RMM	21	22.6
RWS	15	16.1
Categorization of processed products according to their origin		
RS	4	4.3
RR	18	19.4
RF	4	4.3
RP	5	5.4
RSU	5	5.4
MLK	11	11.8
MLU	19	20.4
MU	27	29.0

RU: Ruminant; PO: poultry; FS: fish; PR: pork; UK: unknown; PM: processed meat (salami, sausages, ham, frankfurter, meat ball); RMM: raw mince meat; RWM: raw meat; RS: reference species; RR: raw ruminant; RF: raw fish; RP: raw poultry; RSU: raw but species unknown; MLK: mix, labeled and concentration of species given; MLU: mix, labeled and concentration of species not given; MU: mixed, but species unknown ; #: number of samples; %: ratio of samples.

from control samples, such as quality and quantity, are shown in Table 3. DNA of all samples, which had strength of solution between 44, 75 and 80 ng/ µl were also measured.

Simplex PCR specificity and optimization

To detect possible cross-reactions, each set of primers was performed in simplex PCR with a non-target species. A cross-reaction was not observed in any case. After the simplex PCR was optimized, it was carried out on the DNA samples extracted from raw meat, to reveal the sensitivity and confirm the specificity of the primers (Figure 1). The primers generated specific fragments of 374, 183, 224 and 290 bp for ruminants, poultry, fish and pork, respectively (Figure 2).

Multiplex PCR specificity and optimization

When multiplex PCR was carried out on analogous samples, the set of primers retained the same specificity (Figure 2). The electrophoretic pattern clearly showed the absence of a cross-reaction. In fact, only the species specific band was evident. We optimized the multiplex PCR in the same conditions as those followed for simplex PCR, to check the mixed meat products and show the applicability of multiplex PCR (data not shown) on commercial meat.

Table 2. Design of oligonucleotides of the different animal species.

Primers	Species	Source	Position	Oligonucleotides primers	Amplicon (bp)
Ruminant	*Bos taurus*	16S rRNA-tRNA	*Bos taurus* EU177870	5' GAA AGG ACA AGA GAA ATA AGG 3' 5' TAG CGG GTC GTA GTG GTT CT 3'	374
Pork	*Sus scrofa*	12S rRNA-tRNA Val	*Sus scrofa* bNC 000845	5' CTA CAT AAG AAT ATC CAC CAC A 3' 5' ACA TTG TGG GAT CTT CTA GGT 3'	290
Fish	*Sardinops melanostictus*	12S rRNA	*Sardinops mel.* bNC 002616	5' TAA GAG GGC CGG TAA AAC TC 3' 5' GTG GGG TAT CTA ATC CCA G 3'	224
Poultry	*Meleagris meleagridis*	12S rRNA	*Gallus gallus* bNC 001323	5' GGG CTA TTG AGC TCA CTG TT 3' 5' TGA GAA CTA CGA GCA CAA AC 3'	183

Table 3. The results of quantification and qualification for DNA of control samples.

Control sample	O.D. 260 nm	O.D. 280 nm	O.D. 260/O.D. 280	Concentration (ng/µl)
Ruminant	1.200	0.589	2.037	60
Pork	1.514	0.714	2.120	75.7
Poultry	1.520	0.728	2.087	76
Fish	0.895	0.447	2.002	44.75

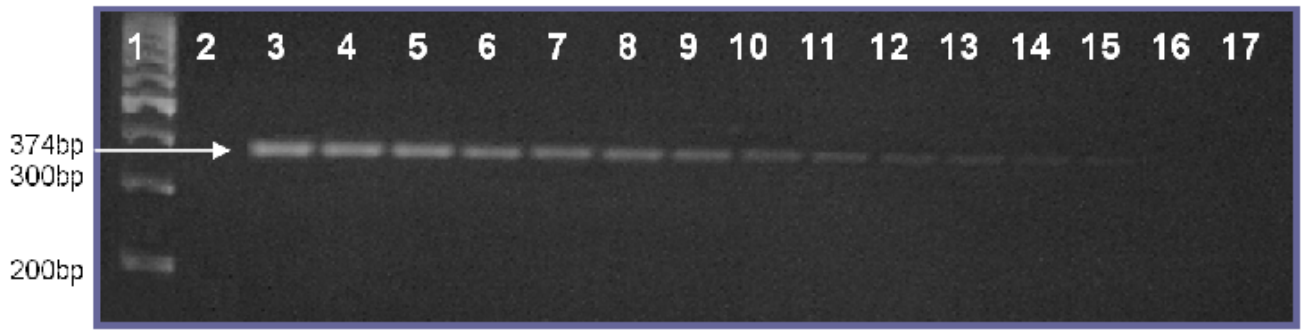

Figure 1. Evaluation of assay sensitivity and progressive dilution of ruminant DNA template, showing diluted in the DNAs of pork, poultry and fish. Lane 1, M, 100 bp ladder; lane 2, control reagent; lane 3, 100%; lane 4, 100%; lane 5, 100%; lane 6, 20%; lane 7, 10%; lane 8, 2%; lane 9, 1%; lane 10, 0.2%; lane 11, 0.1%; lane 12, 0.02%; lane 13, 0.01%; lane 14, 0.002%; lane 15, 0.001%; lane 16, 0.0002%; lane 17, 0.0001%.

The size of the PCR products was as expected, with no additional fragment from a target species. This result showed that, the species-specific primers amplified only one size of fragment from a target species. Primer specificity to the other species was examined by a multiplex PCR using the same primer mixture used in the method. Figure 2 shows the consequence of an optimized multiplex PCR, which resulted in a single band of target size from one meat species and no fragment, was produced by non-specific amplification.

The overall multiplex results showed that 25 (35.1%) among 71 (RU+ (RU+PO) +PO+FS+PR) samples gave unexpected results (Table 4), not indicated in their labels. It was seen that 19 of the 42 RU samples were contaminated with PO, whereas 3 of 10 PO were contaminated with RU. On the other hand, 2 of 42 RU were found as only PO, although, it was stated as only RU on their labels.

We had 30 commercially labeled processed meat products (salami, frankfurter, sausages, etc.) manufactured

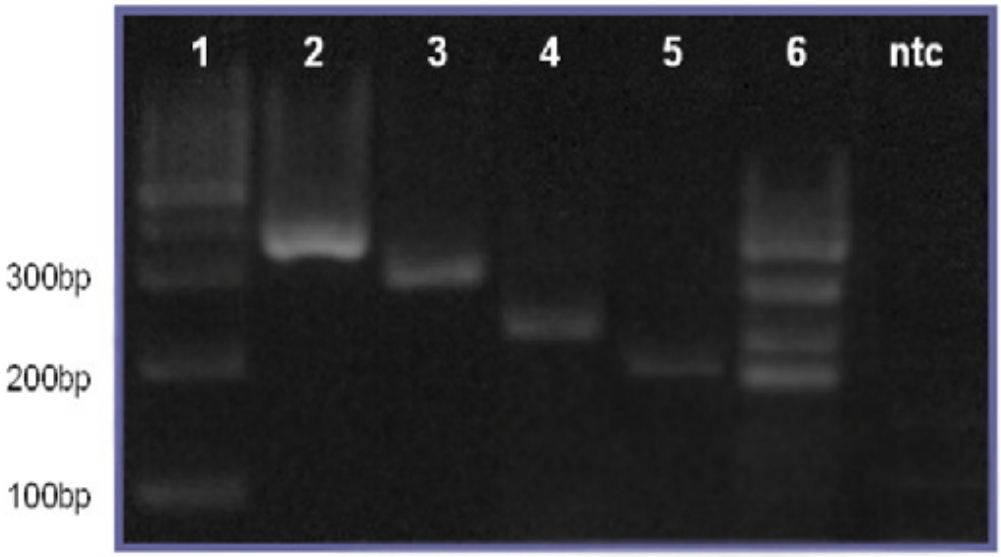

Figure 2. Specificity of M-PCR of DNA isolated from raw meat. Lane 1; Marker, 100-bp ladder; lane 2, ruminant (374 bp); lane 3, pork (290 bp); lane 4, poultry (183 bp); lane 5, fish (224 bp); lane 6, mixture of all animals (ruminant, pork, poultry and fish) DNA; lane ntc, no template control.

Table 4. Overall M-PCR results.

Given species	#	(%)	Revealed results	#	(%)
RU	42	(45.2)	RU	21	(50)
			RU + PO	19	(47.5)
			PO	2	(2.5)
RU + PO	13	(14.0)	RU + PO	12	(92.3)
			PO	1	(7.7)
PO	10	(10.8)	PO	7	(70.0)
			RU + PO	3	(30.0)
FS	5	(5.4)	FS	5	(100.0)
PR	1	(1.1)	PR	1	(100.0)
UK	22	(23.7)	PO	1	(4.5)
			RU	1	(4.5)
			RU + PO	20	(91.0)

RU: Ruminant; PO: poultry; FS: fish; PR: pork; UK: unknown; #: number of samples; %: ratio of samples.

by reputed companies. It was revealed that, one of the manufacturers had used only poultry; another one had used only ruminant meat, whereas, they stated that they used a mixture of poultry and ruminant meat. Overall, it was observed that only 18 of the 30 products conformed to what were declared on the labels. That is, 40% of the commercially labeled products were carrying different meat species which were not indicated in their labels (Table 4).

In addition to ruminant meat, the presence of poultry has been evidenced in many meat products (minced meat, salami, sausage, meat ball, etc.), although, no

poultry meat usage has been given in their official labels. In our experiment, fish and pork meats were not detected in meat and meat products obtained from the market in Istanbul.

DISCUSSION

The M-PCR technique used in the present study depicts the development and application of a multiplex PCR to detect ruminant, poultry, fish and pork materials in processed meat in a single reaction step. This greatly decreases the cost of tests.

We used mitochondrial DNA for the detection of species in processed meat. On account of the high copy number of small, circular mitochondrial DNA in the cells, the chances for their survival under different processing conditions are higher, making it ideal for processed meat species identification (Rudi et al., 2004). Three of the four species-specific primers (12S rRNA, tRNA Val and 16S rRNA) were published by Dalmasso et al. (2004) in order to obtain the amplicon. Sensitivity and specificity of all primers (Table 2) were checked by using the BLAST tool of NCBI. We designed ruminant primers (16S rRNA, accession number: EU177870) using the primer 3 (v. 0.4.0) primer design software (http://fokker.wi.mit.edu/primer3/input.htm), which was accessed on the internet, because the amplicon could not be obtained by the ruminant primer set in Dalmasso's article.

The M-PCR described in this study proved to be very useful when DNA mixtures were tested. The same assay applied on commercial processed meat containing salami, sausage and frankfurter, showed its usefulness in detecting the adulterated meat products. Many investigators have used M-PCR for the same purposes. Dalmasso et al. (2004) applied M-PCR on 13 commercially labeled processed meals (pet food, baby food meat and blood meal). They found that three of the four commercial meals were carrying meat belonging to the species not declared on their labels. Three types of industrial meat products, sausages (N = 10), cold cut (N = 10), and ground meat (N = 10) were tested by Ghovvati et al. (2009) and their results indicated that, none of the samples was contaminated with porcine residuals, but 40% of the sausages samples and 30% of the cold cut samples were contaminated with poultry residuals. In our study, 93 meat products and several types of industrial meat products (Table 1) were analyzed. Overall, the multiplex results showed that, 25 (35.1%) among 71 (RU+ (RU+PO) +PO+FS+PR) samples gave unexpected results (Table 4), not indicated in their labels. It was seen that 19 of the 42 RU samples were contaminated with PO, whereas, three of the ten PO were contaminated with RU. On the other hand, two of the 42 RU were found to be only PO, although, it was stated as only RU on their labels. From another point of view, six and two of the 14 ruminant meat products manufactured by high-class companies were contaminated by poultry and were carrying only poultry meat (Table 4). In our experiments, fish and pork meat could not be detected in meat and meat products bought from markets in Istanbul.

Briefly, our M-PCR assay was applied to raw and processed meats for the identification of commonly used species in foodstuff such as ruminant, poultry, fish and pork materials. Specific species primers, designed in different regions of the mitochondrial DNA were used after alignment of the available sequences in the GenBank database. The primers were the generated specific fragments of 183, 224, 290 and 374 bp lengths for poultry, fish, pork and ruminant, respectively. The optimized M-PCR assay was applied to 93 commercial meat products and it showed the presence of poultry meat in 25/71 of the analyzed products containing raw or processed red meat and indicating the presence of animal species not indicated on the label.

The results of multiplex PCR assay on commercial food have suggested an extension of the assay to other items from the retail trade, such as pet food, baby food and so on. The test could be useful in controlling and verifying the origin of the meat species, especially in products exposed to denaturing technologies (Dalmasso et al., 2004). For instance, the Ouchterlony method cannot distinguish between closely-related species such as wild boar and pig, cattle and buffalo, sheep and goat (Koh et al., 1997). The effectiveness of ELISA and SDS-PAGE is hampered because of denaturation in the food process of species-specific proteins. IEF presupposes that the protein composition of meat is similar within the species and has differences between, for instance, muscle proteins of sheep and goat. However, even the electrophoresis patterns of serum proteins and brain proteins can be different within the same species (Wang et al., 2006).

A clear disadvantage was that M-PCR could not detect whether they (unexpected results) were contaminated or not at the moment these products were manufactured, that is, it could be difficult to establish whether a fraud was presumable or an unintentional contamination that occurred, in the case of very small amounts of contaminating animal materials.

On account of this drawback, we need to develop a quantitative real-time PCR technique by florescence dye or a probe, to quantify the presence of animal material in foodstuff samples. The development and availability of specific quantitative PCR-based methods for the identification of small amounts of DNA are necessary, as a support of an efficient surveillance system because species substitution is lacking at present. The enforcement of legislation guidelines to guarantee public health associated with the improvements of detection methodologies appear to be necessary to differentiate between technically inevitable contamination or intentional

admixture (Pinto et al., 2005).

Conclusions

M-PCR assays applied in this research have a high potential as a molecular tool that can be used in quality control laboratories for the verification and control of contaminated industrial meat products, such as, sausages, salami, meat ball, minced meat and other food, to verify the origin of the raw material. Time for sample preparation is less in M-PCR when compared with other systems where several tubes of uniplex PCR are used. However, if identification of the contamination degree is essential, quantitative real-time PCR can be used.

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