Muhammad SAFDAR

MULTIPLEX ANALYSIS OF ANIMAL AND PLANT SPECIES ORIGIN IN FEEDSTUFFS AND FOODSTUFFS BY MODERN PCR TECHNIQUES: QUALITATIVE PCR AND REAL TIME PCR

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in

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Istanbul, Turkey

APPROVAL PAGE

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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July 2013

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M.S Thesis – Genetics and Bioengineering
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Thesis Supervisor: Assoc. Prof. Dr. M. Fatih ABASIYANIK

ABSTRACT

Recently, European Parliament and the Council of the European Union have implemented very strict rules for the prevention, control and eradication of transmissible spongiform encephalopathies (TSEs). Furthermore, the need of information about the composition of feedstuffs and foodstuffs, in particular of sausages, pet food and ruminant feed has been increased due to health problems, so identifying the species origin is necessary. For this need, DNA-based modern techniques, MQ-PCRs (multiplex qualitative PCRs) and MRT-PCRs (multiplex real-time PCRs) were developed and optimized for the analysis of origin of animal and plant species DNAs in complex feed and food matrix. The primers were generated specific fragments of each species (horse; 85bp, bovine; 93/271/374bp, soybean; 100bp, ovine; 119 bp, caprine; 142bp, pork; 212/ 290bp, Fish; 224 bp) from their respective prepared samples. The optimized MQ-PCR assay was applied to commercial food and feed products and it showed adulterations, not indicated on the labels. Also MRT-PCRs were developed to improve the assay that can combine the two advantages of real-time PCR and multiplex PCR together for animal gene detection quickly. The aim of this study was to design rapid, specific and accurate MRT-PCR assays by using EvaGreen and SYBR Green 1 fluorescence dyes cheaper than double labeled probes to detect animal and plant species simultaneously in food and feed products. Based upon the assays results it has been concluded that multiplex real-time PCR assay might be an efficient tool for the verification of species origin in feedstuffs and foodstuffs submitted to denaturing technologies.

Keywords: Real-time PCR, Qualitative PCR, Multiplex analysis, species identification, Foodstuff, Feedstuff, EvaGreen dye, SYBR Green 1dye.

MODERN PZR TEKNIKLERI KULLANARAK HAYVAN YEMI VE YIYECEKLERE KATILAN BITKI VE HAYVAN ORIJINLERININ ÇOKLU ANALIZI: NITEL PZR VE GERÇEK ZAMANLI PZR

Muhammad SAFDAR

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ÖZ

Yakın zamanda, Avrupa Parlamentosu ve Avrupa Birliği, bulaşıcı süngerimsi ensefalopatinin (TSEs) bulaşmasının önlenmesi , kontrolü ve eradikasyonu için çok sıkı kurallar uygulamaya koymuştur. Buna ek olarak, besin maddeleri ve hayvan yemlerinin, özelikle soslar, evcil hayvan yemleri ve besi hayvanı (geviş getiren hayvan) yemleri, içeriği hakkındaki bilgi ihtiyacı sağlık problemleri sebebi ile artmıştır ve türlerin kökeninin teşhisi gerekli hale gelmiştir. Bu ihtiyaçla, karmaşık besin ve yem matrsindeki, bitki ve hayvan DNAlarının kökeninin analiz edilmesi için DNA- bazlı modern teknikler, MQ-PCR (çoklu quantitatif PZR; *ing:multiplex qualitative PCR*) ve MRT-PCRs (Çoklu Gerçek Zamanlı PZR; *ing:multiplex real-time PCR*), geliştirilmiş ve optimize edilmiştir. Her türün hazırlanan örneklerinden, spesifik boylarda (at; 85bp, sığır; 93/271/374bp, soya fasulyesi; 100bp, koyun; 119 bp, keçi; 142bp, domuz; 212/ 290bp, balık; 224 bp) primerler oluşturuldu. Optimize edilen MQ-PCR testi ticari besin ve yem ürünlerinde uygulanmış ve etiketlerde belirtilmeyen uygunsuzlukların olduğu görülmüştür. Ayrıca, hayvan geni tesbitinin kısa sürede yapılabilmesi için gerçek zamanalı PZR ve çoklu PZR tekniklerinin iki avantajını birleştirebilen MRT-PCRs geliştirilmiştir. Bu çalışmanın amacı, çift taraflı işaretli problardan daha ucuz olan EvaGreen and SYBR Green 1 flüoresan boyaları kullanarak, besin ve yem maddelerindeki hayvan ve bitki türlerini aynı anda tayin etmeyi sağlayan, hızlı spesifik ve hassas MRT-PCR tahlilleri dizayn etmektir. Bu tahlillerin sonuçlarına dayanarak çoklu gerçek zamanlı PZR tahlilinin besin maddeleri ve yemlerdeki türlerin kökeninin doğrulanması için etkili bir araç olabileceği sonucuna varılmış ve denatüre teknolojilere sunulmuştur.

Anahtar Kelimeler: Gerçek Zamanlı PZR, Quantitatif PZR, çoklu analizi, tür teşhisi, besinmaddesi, yem, EvaGreen boya, SYBR Green 1boya

To my lovely parents

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

TSE	Transmissible spongiform encephalopathy
BSE	Bovine spongiform encephalopathy
vCJD	variant of Creutzfeldt–Jakob disease
PCR	Polymerase chain reaction
MQ-PCR	Multiplex Qualitative PCR
RT-PCR	Real-time Polymerase chain reaction
MRT-PCR	Multiplex Real-time PCR
SDRT-PCR	SYBR Green Duplex Real-time PCR
SMRT-PCR	SYBR Green Multiplex Real-time PCR
EMRT-PCR	EvaGreen Multiplex Real-time-PCR
EU	European Union
MBM	Meat and Bone Meal
Mt DNA	Mitochondrial DNA
FDA	Food and Drug Administration
ELISA	Enzyme-linked immunosorbent assay
IEF	Iso-electric focusing
RAPD-PCR	Random amplified polymorphic DNA fingerprints
Tm	Melting temperature
LED	Light-emitting diode
dF/dT	Function of time versus temperature of the reaction in celcious
NCBI	National Center for Biotechnology Information
Cyt b	Cytochrome b

CHAPTER 1

INTRODUCTION

It is a biologically known fact, that right since the first living organism breathed for the first time billions of years ago, it needed food. Food is something without which growth, development and evolution would have been impossible. Every living thing on the face of the earth, irrespective of plants and animals, need nutrition to survive, grow and reproduce. Without a catalyst, there is no product that is formed and for all living things like plants, animals and humans, food is the catalyst. Hence, when you consume food, nutrition is provided to the body for the production of energy and in turn, the body is functional. Food which is given to domestic animals in the course of animal husbandry is called feed.

The detection of animal and plant species in feedstuffs and foodstuffs is needed to be performed for various reasons, which includes Transmissible spongiform encephalopathy (TSE), illegal substitution of ingredients with cheaper one, religious, health reasons and conservation regulations etc. Bovine spongiform encephalopathy (BSE), commonly referred as ‘‘mad cow disease,’’ has a human form termed vCJD (variant of Creutzfeldt–Jakob disease) which is a fatal neurodegenerative disease that has caused large in number deaths [1]. Currently, consumers want to buy quality products which are labeled well. However, false or accidental mislabeling still exits and may not be detected, resulting in poor-quality products.

The identity of the ingredients in processed feedstuffs and foodstuffs are not always readily apparent and verified that the components are authentic and from sources acceptable to the consumers should be required. In most countries, feed and food manufacturers choose to use some products instead of another one such as lard as a

substitute ingredient for oil because they are cheaper and easily available. Biological complications and health risks may be associated with daily intake. Hence, it is an important task for feed and food control laboratories to be able to carry out species differentiation of raw materials to be used for industrial feed and food preparation and the detection of animal and plant species in feed and food products [2]. Ultimately, the species identification from mixed feedstuffs and foodstuffs are very critical. However, it is not always possible to differentiate the species by currently available laboratory methods.

The conventionally available methods for species identification from feedstuffs and foodstuffs include various forms of electrophoresis and use of immune sera in agar gel diffusion. Some of such methods of animal tissue identification are agar diffusion, passive haemagglutination, immuno-electrophoresis, enzyme-linked immunosorbent assay and counter immunoelectrophoresis etc. Although electrophoretical and chromatographic methods are very popular, they have many disadvantages such as difficult optimization procedures, time consuming, high cost and low specificity and sensitivity. On the other hand, the use of antisera to thermostable antigens has proved to be superior in identification of animal species in heated foodstuff and feedstuff. However, use of such antigens and antisera against them are only partially successful in identification of closely related species of animals like cattle and buffalo from sheep and goats [3].

Now a days, DNA as a source of information has been used for speciation of animal and plant species. DNA based technology for such purpose has several advantages such as:

- DNA have significant amounts of sequence variation in closely related species
- DNA is more thermostable than many proteins
- DNA is easier to retrieve from low-quantity and/or degraded DNA from samples
- Small amount of template DNA is required for identification of species

Two major approaches to identify species in feedstuffs and foodstuffs by DNA techniques are DNA hybridization and PCR based methods. DNA hybridization was the first genetic approach for determination of species identity. In this method, labeled DNA probes were hybridized to samples of genomic DNA covalently attached to nylon membranes in a slot or dot blot form [4,5]. It was observed that the probes comprising

labeled total genomic DNA from a given species would hybridize to DNA from the same species with little cross reactivity. The technique of DNA hybridization has been successfully applied for identification and differentiation of species in commercial food products [6,7]. However, closely related species of animals, like sheep and goats showed cross reactivity by this method. That's why; DNA hybridization techniques are complicated and inadequate.

PCR is a promising approach to species identification in feedstuffs and foodstuffs. This method is easy, fast and more sensitive. A number of strategies have been employed in PCR including use of repetitive sequences [8], multi-gene family [9] and use of mitochondrial gene [10] for species identification.

In feed and food technology, there is always a possibility for products to be contaminated with minute quantity of ruminants, fish, pork, horse etc. It results in a requirement to develop qualitative and quantitative DNA based method that exactly distinguish feed and food samples containing low level of contamination from ones that are deliberately mis-described or adulterated.

In contrast to normal PCR, real-time PCR, are able to differentiate and measure even minute traces of different animal species in feedstuff and foodstuff. Real-time data collection is accomplished by fluorescent molecules providing a strong correlation between fluorescence intensity and PCR product concentration. The fluorescent based methods used in real time PCR can be classified in two categories: probe-based such as TaqMan and DNA intercalating dyes such as the EvaGreen and SYBER Green. Although probe-based ones are more accurate and additionally sequence- specific, but they are more expensive, time consuming and intensive use of labor. It is also much more difficult to design and optimize them. That is why EvaGreen or SYBER Green is used to detect PCR products during real-time PCR without any probes. Optimization is easy because the protocols in use for normal PCR can be used with only slight modifications. Economically, it is almost seven folds cheaper than probe-based methods.

Şakalar E. developed conventional multiplex PCR and duplex real-time PCR for the analysis of meat species origin and quantification in meats products [11]. EMRT-PCR (Eva Green Duplex Real-time-PCR) assays applied on the poultry and pork DNAs

in pet food products using Eva Green florescence was first one in the field of molecular food analysis [12]. Through EMRT-PCR technique, we did not apply on other animal species in foodstuffs and feedstuffs yet. Also nobody has applied EMRT-PCR (EvaGreen Multiplex Real-time-PCR) on feedstuffs and foodstuffs yet. For this reason, the present study was undertaken specifically to develop a diagnostic test useful for an advanced laboratory to differentiate species in feedstuffs and foodstuffs by all techniques; Qualitative Multiplex PCR, EvaGreen real-time multiplex PCR and SYBER Green real-time multiplex PCR. The study was carried out with the following objectives.

(Part1) Rapid multiplex analysis of animal and plant species origin in feedstuffs and foodstuffs by qualitative PCR

(Part2) Rapid multiplex analysis of animal and plant species origin in feedstuffs and foodstuffs by Real-time PCR using EvaGreen and SYBER Green florescence dyes

CHAPTER 2

REVIEW OF LITERATURE

2.1 FEEDSTUFFS AND FOODSTUFFS

2.1.1 Potential Problems of feedstuffs and foodstuffs

Transmissible Spongiform Encephalopathies (TSEs) are fatal neuro-degenerative diseases which refered as bovine spongiform encephalopathy (BSE) or ‘‘mad cow disease,’’ in bovine, variant of Creutzfeldt–Jakob disease in human [1] and scrapie in sheep and goat [13]. To minimize the risks of TSEs to humans and animals, the European Food Safety Authority adopted measurment to restrict the fish meal directly or indirectly in ruminant feed [14]. It is strongly suspected to have arisen from feeding cattle with rendered protein supplements derived from scrapie-infected sheep and goat tissues and its spreading strictly correlated with the absence of stringent control on rendering processes [15]. After putting ban on cattle feed enriched with ruminant derived protein, contaminated with infected material and intraspecies recycling has decreased the BSE incidence in many countries. Similarly, the European Union (EU) has introduced restrictions in the production and use of meat and bone meal in farm animal’s feed [16, 17, and 18]. Later on the Annexe IV in Regulation 2003/1234/EC amended the TSE Regulation, in the sense that all animal proteins from farmed animals are prohibited for the use in feedstuffs of farmed animals, due to the lack of animal-specific detection methods.

Wrong labeling of animal foods represents not only a commercial fraud but also a potential health problem in the case of consumers who exhibit sensitivity to undeclared allergens [19]. It is now clear that beef allergy is not an infrequent occurrence, with an incidence between 3.28% and 6.52% among children with atopic dermatitis and its incidence may be as much as 0.3% in the general population [20]. Food allergies affect

up to 2% of the adult population and up to 8% of children [21]. Among food allergies, lupin and soya allergies are becoming an important public health concern because it affects an increasing number of children and adults in Europe [22].

Hindu populations also choose not to eat beef, while Jewish and Muslim populations choose to avoid consumption of pork, even in minute quantities, due to their religious beliefs. Many consumers prefer to include more chicken in their diet instead of beef, sheep or pork. In addition to infectious disease and religious concerns, many individuals are altering their eating behavior to include more chicken simply to reduce dietary fat intake in accordance with health trends. Any conceivable ambiguity in the labeling practices of commercial suppliers or grocery stores is unacceptable to these populations. The need for sensitive detection and quantification of bovine, chicken, porcine, and species in food and mixed foods products is critical in response to this consumer demand. According to Izmir Veterinary Presidency's analyses of a dish made of minced meat (çiğ köfte) which must be prepared with cow meat other meats (pork, horse and donkey meat) were detected [23].

2.1.2 Legislation for feed and food products

In response to the BSE epidemic in Europe, the United States Food and Drug Administration (FDA) imposed strict guidelines in 1997, prohibiting the use of ruminant-derived protein in the manufacture of animal feed intended for cows or other ruminants [1].

The European Union has implemented a set of very strict procedures for the labeling of food/feed. Throughout the whole legislative procedure, the EU ensures the European consumers' right to be fully informed. Thus analytical methods for the species detection of food/feed are essential in order to verify compliance with labeling requirements [24].

In Turkey, compound feed are regulated by the Feed Law no. 1734 and Implementing Regulation on Feed. According to the National Feed Legislation, only approved establishments with qualified personnel are allowed to produce and market compound feedstuffs. Labeling rules are similar to EU Directive 79/373/EEC. Compound feedstuffs should be placed on market in sealed packages or containers. They may be delivered in bulk or unsealed containers only in case of direct deliveries

from producer to final user. Compound feed establishments are inspected by the competent authority twice a year according to the Feed Law. Feed materials are categorized by the Feed Law and Implementing Regulation on Feed. Feed materials have to be genuine, sound and in merchantable quality with the accompanying documents providing their names, quantity, name and address of seller or distributors [25].

In Turkey, the species' which are used to prepare the food products have to be presented on the label of product. Moreover, selling the other species foods with different labels to get more profit is held as imitation and prohibition according to the Foodstuff Laws [25].

2.1.3 Methods for Feed and food Origin Analysis

Methods to analysis feed products can be divided to three categories according to the type of the target they focused on.

- Protein-based Methods
- DNA-based Methods

2.1.3.1 Protein-Based Methods

Many analytical methods that rely on protein analysis have been developed for identification of species such as liquid chromatography [26] electrophoresis techniques [27] immunological methods such as the enzyme-linked immunosorbent assay (ELISA) test[28, 29] Isoelectric focusing (IEF) [30], glycogen levels in the muscle tissue, electrophoretical profiles of sarcoplasmic proteins. But these methods have been failed to analyze complex feed and food matrices due to denaturation of protein at high temperature. Therefore, it appears that DNA-based methods are more accurate.

2.1.3.2 DNA-Based Methods

Two major approaches to identify species in feeds by DNA techniques are DNA hybridization and PCR based methods. The technique of DNA hybridization has been successfully applied for the identification and differentiation of animal species in

commercial products [6]. However, closely related species of animals, like sheep and goats showed cross reactivity by this method. Further, the method is time consuming and hazardous. PCR is a promising approach to species identification which is easy, fast and more sensitive.

PCR, a method for amplification of DNA in an artificial environment, has been successfully used for species identification of animals, plant and bacteria [31]. The dot-blot technique was the first genetic approach for determination of species identity [32, 33]A method of DNA analysis have been developed to verify authenticity of labeled raw material of canned fish or in products from closely related fish species (tuna, eel, salmon, trout and sturgeon) [34]. Short segments (123-358 bp) of the mt cyt b gene were amplified by the PCR and analyzed by electrophoresis to get species specific patterns of ssDNA. DNA strands were separated by polyacrylamide gel electrophoresis and visualized by silver staining [35].

Asensio et al. [36] developed a method of DNA analysis to verify the authenticity of grouper *(Epinephelus guaza),* wreck fish *(Polyprion americanus),* and Nile perch *(Lates niloticus)* fillets. A short fragment (208 bp) of the mt 12S rRNA gene was amplified by the PCR and analyzed by SSCP to get species-specific patterns of ssDNA.

At present; however, polymerase chain reaction (PCR) is an important technique for species identification [37]. Some PCR approaches are RAPD-PCR (random amplified polymorphic DNA fingerprints) [38], DNA mitochondrial D-loop analysis and RFLP analysis of different PCR fragments [26, 39]. DNA hybridization methods are complicated and generally inadequate, but PCR easily amplies target regions of template DNA in a much shorter time and thus it is suitable for different species identification.

Matsunaga et al. [39] developed a simple method using multiplex PCR for simultaneous identification of six meats. Polymerase Chain Reaction (PCR) has been applied for the detection of bovine tissue in animal feedstuffs [40, 41]. Lahiff et al. [42] developed a PCR to identify ovine, porcine and poultry DNA in feedstuffs. Meyer et al and Lahiff et al. [26, 42] identified different species in feedstuffs by using universal primers coupled with restriction endonucleases. Moreti et al. [43] described a DNA monitoring method to examine fishmeal for contamination with mammalian and poultry

products. A PCR method based on the nucleotide sequence variation in the 12S ribosomal RNA gene of mt DNA was developed and evaluated. Three species-specific primer pairs were designed for the identification of ruminant, pig, and poultry DNA. The specificity of the primers used in the PCR was tested by comparison with DNA samples for several vertebrate species and confirmed. The PCR specifically detected mammalian and poultry adulteration in fishmeals containing 0.125 % beef, 0.125 % sheep, 0.125 % pig, 0.125 % chicken and 0.5 % goat. A multiplex PCR assay for ruminant and pig adulteration was optimized and had a detection limit of 0.25 %.

Chapman et al. [44] developed a multiplex PCR assay utilizing both nuclear and mt cyt b gene loci simultaneously for accurate identification of white shark body parts, including dried fins. Cheng et al. [45] developed a PCR assay to identify bovine, porcine, ovine, and chicken meat and bone meal in animal diets. Pinto et al. [24] optimized duplex PCRs in order to identify animal species in food stuffs.

Ghovvati et al, Zorica et al. [48, 49] made a triplex PCR assay for identification of different animal and plant species. Dalmasso et al. [50] developed sensitive multiplex PCR. The detection limit was 0,004% for fish primer and 0,002% for ruminants, poultry and pork primers. Four pairs of primers that targeted highly conserved regions of mt DNA were used. These gene fragments at the targeting region for the four species were 271 bps, 225 bps, 212 bps and 266 bps in size. The PCR products were digested with restriction enzymes *Hph I*, *Mn II*, *Ssp I*, and *Hind III*, and different length polymorphisms were observed. Marıa Lopez-Andreo et al. developed six TaqMan real-time polymerase chain reaction (PCR) systems using minor groove binding (MGB) probes for the detection quantitation of bovine, porcine, lamb, chicken, turkey, and ostrich DNA in complex samples. Koppel et al. [51] developed heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. Species-specific primers and probes were used. The limit of sensitivity of this test was 2%. A. Benedetto et al. [52] developed a real-time PCR method to detect fish DNA in feedstuffs was developed and optimized. A combination of primers and a Taqman-MGB probe was used to selectively amplify the fish mitochondrial 12S ribosomal RNA gene. The limit of detection was 0.2 pg of fish DNA in feedstuffs.

2.2 POLIMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA thus generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations [53].

PCR is a recently developed procedure for the *in vitro* amplification of DNA. Developed in 1983 by Kary Mullis, PCR is now a common and useful technique used in medical and biological research labs for a variety of applications [54]. The first report related to PCR was in 1985, more than 5000 scientific papers were published by 1992 [55]. In 1993, Mullis was awarded the Nobel Prize in Chemistry for his work on PCR. Previous techniques for isolating a specific piece of DNA relied on gene cloning slow procedure and a tedious PCR. When other scientists eventually succeeded in making the polymerase chain reaction perform as desired in a reliable fashion, they had an immensely powerful technique for providing essentially unlimited quantities of the precise genetic *material* [56].

2.2.1. General Principles of the PCR

The vast majority of PCR methods use thermal cycling defined as alternately heating and cooling the PCR sample to a certain series of temperature steps. PCR relies on thermal cycling consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs. It thus can amplify a specific sequence of DNA by as many as one billion times. As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the

sequences between the PCR primers, which are finally represented at a theoretical abundance of 2n, where n is the number of cycles [55, 56, 57].

2.2.2 PCR Steps

2.2.2.1 Initialization Step

This step consists of heating the reaction to a temperature of 94-96 or 98°C if extremely thermostable polymerases are used, which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR. Hot-start PCR is a technique that reduces non-specific amplification during the initial set up stages of the PCR [58].

2.2.2.2 Denaturation Step

This step consists of heating the reaction to 94-98 °C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA [59]. As Andy Vierstraete designed following reactions.

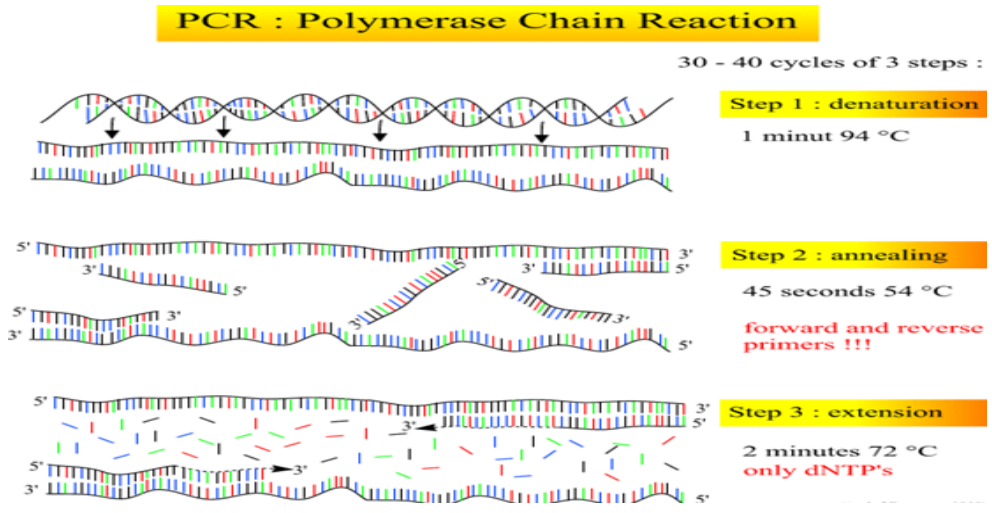


Figure.2.1 PCR different steps Amplification Mechanisms

2.2.2.3 Annealing Step

In this step, the mixture is cooled to a temperature of 50–65 °C for 20-40 seconds which helps in annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence that permits annealing of the primer to the complementary sequences in the DNA. Since the primer concentration is kept very high relative to that of the template DNA, primer-template hybrid formation is greatly favored over re-annealing of the template strands [59].

2.2.2.4 Extension/Elongation Step

Almost all PCR applications put to use a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80^{o}C and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's that are complementary to the template in 5' to 3' direction. The extension time not only depends on the DNA polymerase used but also on the length of the DNA fragment to be amplified. The DNA polymerase polymerizes a thousand bases in per minute at its optimum temperature [60].

2.2.2.5 Final Elongation

This single step is performed at a temperature of 70-74^{o}C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended [61]

2.2.2.6 Final Hold

This step has performed at 4-15^{o}C for an indefinite time may be used for short-term storage of the reaction [61].

2.3 MULTIPLEX QUALITATIVE PCR (MQ-PCR)

2.3.1 Advantages of MQ-PCR

Multiple targets are amplified simultaneously with different primers in one PCR reaction. The resulted products with differential sizes are easily distinguished with regular agarose gel electrophoresis (see Figure 2.2). The parameters of PCR including the primer concentration and the reaction buffer are optimized in order to provide the highest specificity and sensitivity of amplification of multiple targets in one reaction.

Multiplex-PCR was first applied in 1988 as a method to detect deletions in the dystrophin gene [62]. In 2008, multiplex-PCR was developed for analysis of microsatellites and SNPs [63].

By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work properly within a single reaction, and amplicons sizes, i.e., their base pair length, should be different enough to get bright bands when visualized by gel electrophoresis [64].As this picture designed by Signosis Inovative Plate Assay Solutions company.

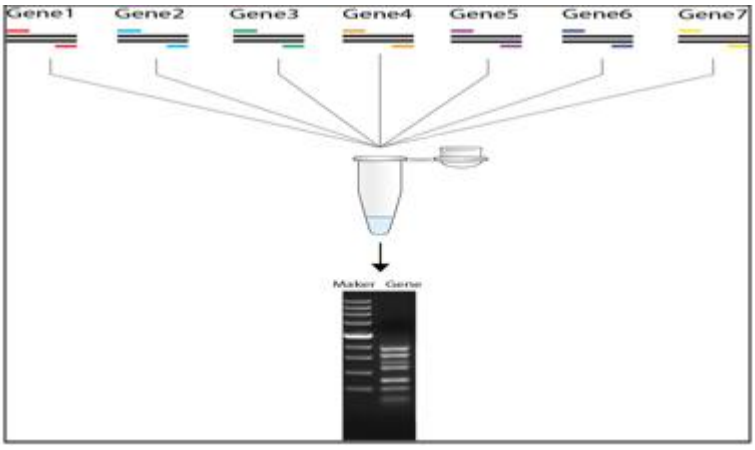


Figure.2.2 Identification by Multiplex PCR Kits.

2.3.2 Parameters for Primer Design (Multiplex PCR)

The essential primer design considerations has been described below which can be key to specific amplification with PCR products.

2.3.2.1 Primer Length

Multiplex PCR requires well designed primer with appropriate length. Usually, short primers (18-23 bases) are used.

2.3.2.2 Melting Temperature

Preferable melting temperature (Tm) values for such primers are between 55^{o}C - 60^{o}C. For sequences with high GC content, primers with a higher Tm (preferably 75^{o}C - 80^{o}C) are recommended. Tm variation is suitable between 3-5^{o}C for primers used in a reaction [61].

2.3.2.3 Specificity

It is important to consider the specificity of designed primers to the target sequences, while preparing a multiplex assay because of presented competition when multiple target sequences are in a single reaction tube. If designed primer has not sufficient specificity to the target DNA sequence in multiplex reaction, it is complementary to the other target sequences and it leads to imprecise results [61]

2.3.2.4 Avoid Primer Dimer Formation

The designed primers in the same reaction tube should be checked for formation of primer dimers. Dimerization leads to non specific bands [61].

2.4 REAL-TIME PCR

2.4.1 History of Real-Time PCR

PCR has become even more well-liked with the introduction of real-time PCR. Simultaneous amplification and revealing of specific DNA sequences in real-time by

simply adding ethidium bromide (EtBr) to the PCR reaction is first established by Higuchi and colleagues so that the accumulation of PCR product could be visualized at each PCR amplification cycle. When EtBr is bound to double-stranded DNA and poured by UV light fluorescence. An increase in fluorescence in a PCR assay indicates positive amplification. After that they introduced the idea of real-time PCR product quantization by constantly measuring to add in EtBr intensity during amplification with a charge-coupled device camera [65]. By creating amplification plots of fluorescence increase versus the cycle number, they showed that the kinetics of EtBr fluorescence accumulation during thermo-cycling was openly related to the starting number of DNA copies [66].

Fewer cycles are desired to create a clear signal when a greater number of target molecules are available. Real-time monitoring also provided to decide the efficiency of amplification under different conditions in PCR processes. The principle of real-time PCR can simply be declared as the monitoring of fluorescent signal from one or more PCRs cycle-by-cycle to completion, where the total quantity of product produced during the exponential amplification stage can be used to find out the amount of preliminary material [67].

The approach described above was not ideal because EtBr binds nonspecifically to DNA duplexes and non-specific amplification products, such as primer–dimers, can give to the fluorescent signal and result in quantification inaccuracies. Subsequent investigations, fluorogenic probes were introduced to check PCR product accumulation that could increase the specificity of product in real-time PCR and gave greater quantitative accuracy and dynamic range than previous techniques [78].

2.4.2 Development to Real-time PCR

In molecular biology, real time PCR or quantitative real time polymerase chain reaction (qPCR) is a kind of PCR technique that is used to amplify and simultaneously quantify a targeted DNA molecule. The method follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is recognized as the reaction progresses in real time. For more than one specific primers of different species in multiple DNA sample, Real Time-PCR enables both identification and quantification [69].

In real-time PCR the quantity of PCR product is checked during the reaction by monitoring the fluorescence of dyes or probes that is relative to the amount of product formed, and the number of amplification cycles required to obtain a specific amount of DNA molecules. The number of DNA traces of a particular sequence in a compound sample can be determined with high accuracy and sensitivity by the help of the highly efficient detection chemicals, sensitive instruments, and optimized assays [70].

This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Real-time PCR can be divided into two different types according to the style of PCR product detection: (1) DNA Intercalating dyes such as EvaGreen, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which allows detection only after hybridization of the probe with its complementary DNA target [71]. Real-Time chemistries like chemicals and dyes allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR gives a distinct advantage over Normal/conventional detection. Normal methods use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. End point detection is very time consuming. Perhaps the most important advantage is its ability to quantify nucleic acids over a particularly wide dynamic range (at least 5 log units). This is fixed to tremendous sensitivity, allowing the detection of less than five copies of a target sequence, making it possible to analyze small samples. In addition, all real-time platforms are relatively quick, with some affording high-throughput computerization [72].

The major disadvantage of real-time PCR is that it requires expensive equipment and reagents. In addition, due to its extremely high sensitivity, experimental probe design and optimized for accurate conclusions [69].

2.4.3 Real-time PCR Analysis

Real-time PCR also needs a fluorescent reporter that binds to the PCR product formed and reports its presence by fluorescence (see Figure. 2.4). The reporter produces a fluorescence signal that reflects the amount of PCR product formed. During the initial cycles the signal is weak and cannot be differentiated from the setting (see Figure. 2.5). As the amount of PCR product accumulates a signal develops that initially increases

exponentially. After that the signal levels off and saturates. The signal saturation is due to the reaction running out of some significant component. This can be the primers or the reporter. Also the number of polymerase molecules may be limiting, in which the exponential amplification goes over to linear amplification. It is important note that in a typical real-time PCR experiment, all response curves saturate at the same level [73].

2.4.4 Fluorescent Reporters of Real-time PCR

Nowadays fluorochromes are used as the detection materials in real-time PCR. Both sequence specific probes and non-specific intercalating dyes are available as reporters.

2.4.4.1 Intercalating DNA Binding Dyes

Classical intercalators interfere with the polymerase reaction, and a symmetric cyanine dyes such as SYBR Green I and EvaGreen are become more popular. (see Figure. 2.2). Asymmetric cyanine shave two aromatic systems containing nitrogen, one of which is positively charged, connected by a methane bridge [74, 75].

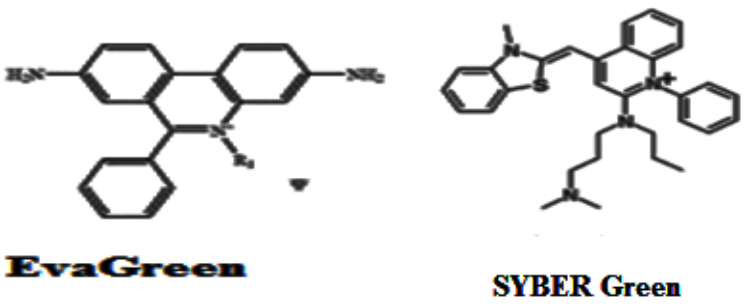


Figure 2.3 Asymmetric cyanine dyes SYBR Green 1 and EvaGreen.

Although probe-based chemistries provide additional sequence-specificity to the PCR primers, it is commonly difficult to design and optimize them and also costs of these analysis is very expensive. In contrast, a DNA binding dye such as SYBR green and EvaGreen, which adheres to the minor groove of the double stranded DNA in a

sequence-independent way, provides a flexible technique without the need for any individual probe design and optimization steps [76].

The advantages of SYBR Green are as follows: inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green can be binded nonspecifically to any double-stranded DNA in the reaction including primer dimers and other nonspecific reaction products resulting in an over estimation (false positive result) of the target concentration[77].

Herein, the critical point is to recognize whether it is the correct amplification or not. This can be confirmed by melting curve analysis after completing the PCR. EvaGreen and SYBR Green can work extremely very well for simplex PCR with well-designed primer set [78].

2.4.4.2 Specific Probes

Commercial probes have been done with different chemistries are available for real time detection such as Taqman probes [79], Molar Beacons, Hybridization probes , Scorpion probes (see Figure 2.4).

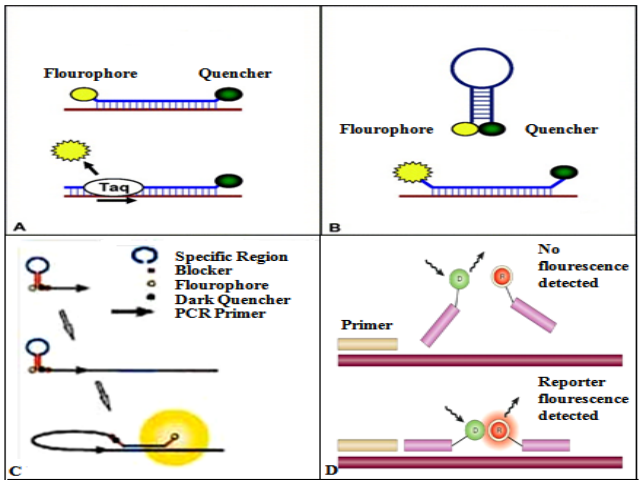


Figure 2.4 Mechanisms of flourescence probes used in real-time PCR: (a) Taqman probe, (b) Molecular beacon, (c) Scorpion probe, (d) Hybridization probe.

2.4.5 Real-time PCR Instruments

A major requirement for real-time PCR machinery is the ability to detect the fluorescent signal and verify the progress of the PCR. Since fluorochromes need not only a specific input of energy for excitation but also a detection of a particular emission wavelength, the instrumentation must be able to do both simultaneously at required wavelengths. Thus the fluorochromes and instrumentation are linked. Edwards et al. described basic ways in which real-time instrumentation can deliver the excitation energy for fluorophores: by lamp, light-emitting diode (LED), or laser (see Figure 2.5)

In figure 2.4, samples are placed in a real-time PCR machine. The samples are exposed to excitation energy, and the resulting fluorescence is measured by a photo detector with each cycle.

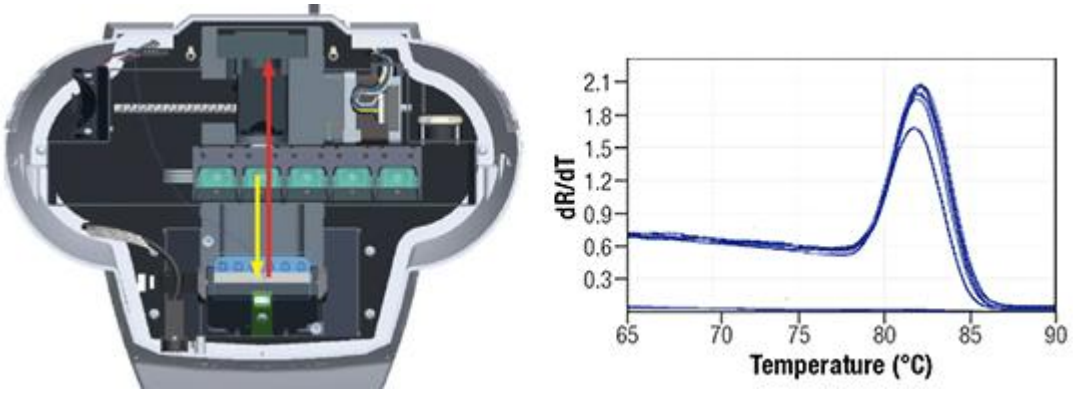


Figure 2.5 Cross-section of reaction chamber and appearance of curves of application.

2.4.6 Melting Curve Analysis

The real-time machine not only monitors DNA synthesis during the PCR but also determines the melting point of the product at the end of the amplification reactions. Melting curve analysis is cleared as an assessment of the dissociation-characteristics of double-stranded DNA during heating. The melting temperature of a DNA double helix depends on its bases composition. All PCR products for a particular primer pair should have the same melting temperature - unless there is mis-priming, primer-dimer, artifacts, contamination or other problems. Nygren et al. told that SYBR green does not

distinguish between one DNA and another, melting curve analysis is checked that all samples have a similar melting temperature.

Following real time PCR amplification, the machine is programmed to do a melt curve. To carry out melting curve analysis, the temperature is increased very slowly from a low temperature (e.g., 65^{o}C) to a high temperature (e.g., 95^{o}C). At low temperatures, all PCR products are double stranded, so SYBR Green I dye binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured resulting in fast decreases in fluorescence (see Figure 2.6). Ririe et al. said that the melting temperature is determined as the maximum of the negative first derivative of the melting curve. Because primer–dimer products typically are shorter than the targeted product, they melt at lower temperature and easily recognized by melting curve analysis.

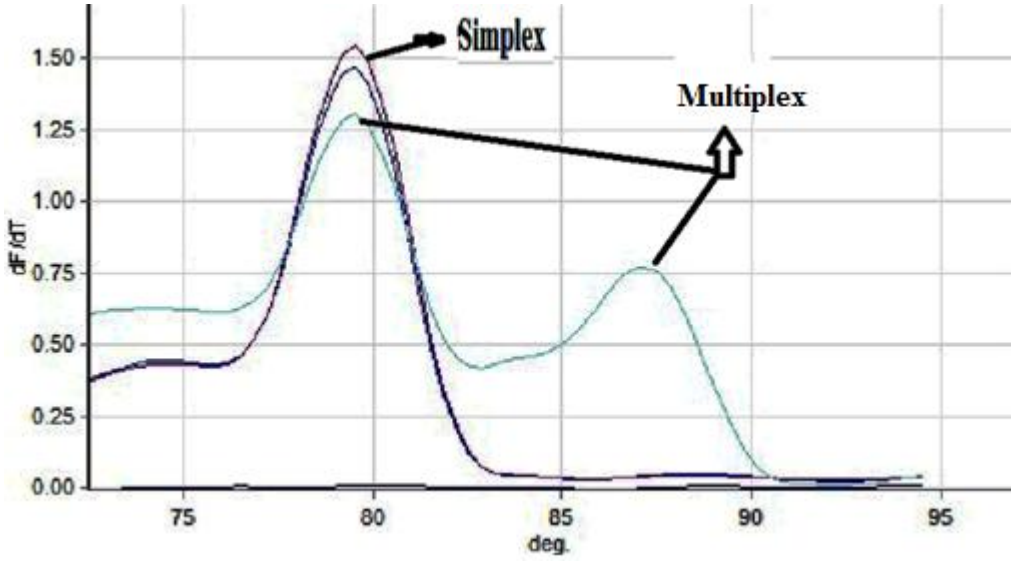


Figure 2.6 Simplex and multiplex real-time PCR analysis in same reaction.

In Figure 2.7, dye fluorescence drops quickly when the DNA melts. The target product amplicon is typically longer and melts at higher temperature than the primer–dimers.

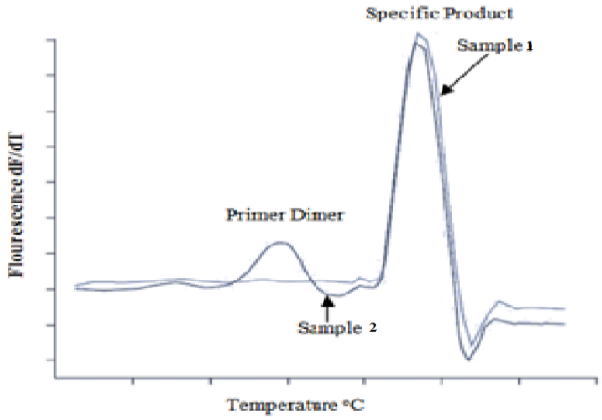


Figure 2.7 Melting curve analysis of two samples (1 and 2). Sample 1 yields only one peak resulting from the specific amplification product (primer-dimers not co amplified). Sample 2 shows a peak from the specific product and a peak at a lower temperature from amplification of primer-dimers.

2.5 MULTIPLEX REAL-TIME PCR (MRT-PCR)

Wei Fan et al. descried the Real-time PCR assay gave analogous sensitivity and superior reproducibility, precision and shorter performance time when compared to earlier methods. This fluorescence-based real-time assay not only can quickly detect target genes separately but also allows for multiple PCR reactions in one tube by employing the exceptional melting curve analysis following DNA amplification.

Hein et al. told that Duplex Real-time PCR allows amplification two target genes simultaneously within a single reaction tube using two primers. The templates are amplified in the Real-time PCR followed by melting curve analysis. It is absolutely vital that results got from multiplex reactions are demonstrated to confirm that the same results would be obtained if the reactions were performed individually.

When designing and choosing Real-time PCR primers for multiplex assays, it is important to judge the following:

- A primer should not include bases that are complementary to the other bases (primer) like self-complementary or complementary to other primers and form primer dimers.

- 40–60% GC content is suggested for all primers, avoiding long stretches of any one base.
- At least 3^{o}C should present between the melting temperatures of target DNAs in order to differentiate peaks in melting curve.
- Annealing temperatures of primers should be closed to each other for PCR optimization step.
- The length of the amplicons should not be very long for optimal PCR efficiency. Primers that generate a longer amplicon may result in poor amplification efficiency.
- The primer should be specific for the target. Perform a BLAST.

Multiplex real-time PCR represents the change in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. Since SYBR Green fluorochrome effectively is bound all amplicons without establishing a direct differentiation between cow, soybean, poultry, horse, pork sheep, goat etc specific products, multiplex RT-PCR fragments are detected by melting curve analysis. Hence, cow, soybean, poultry, horse, pork amplicons can be easily distinguished by specific Tm values due to the different length and compositions of two amplicons.

CHAPTER 3

EXPERIMENTAL PART

3.1 MATERIALS

3.1.1 Feedstuffs and foodstuffs Samples

During this research, some food and feed samples were made in our laboratory and others were collected from the markets.

Table 3.1 Samples submitted to the assays.

Samples	Species
Pure Ruminants meats	Bos taurus
	Capra hircus
	Ovis aries
Pure Fish meats	Sardinops melanosticus
	Oncorhynchus mykiss
	Scomber scombrus
Pure Pork meat	Sus scrofa
Pure Horse meat	Equus caballus
Pure Poultry meat	Meleagris meleagridis
Pure Soybean	Glycine max
Pure Maize	Zea mays
Baby foods, n® : 20	Soya, mutton, beef
Autoclaved meats, n® : 30	mixed
Sausages, n® : 50	mixed
Salami, n® : 30	mixed
Meat ball, n® : 30	mixed
Pet foods, n® : 20	Lamb
Pet foods, n® : 12	Fish

Table 3.1 (cont.)

Pet foods, n® : 15	Beef
Ruminants feed, n® :50	nat

nat: no animal tissues in these samples
n® : number of samples
mixed : cow, sheep, goat, soya, pork, poultry.

3.1.2 Equipments

The list of the machines and equipments used in the study was shown below.

Thermo cyclers :TECHNE TC-512(UK), Corbet Rotor-Gene 6000 , Rotary Analyzer (AUSTRALIA)

Software : Rotor-Gene Software

Autoclave : CERTO CLAW A-4050 Traun, Austria

Camera : Sony Cyber shot DSC T520

Centrifuges : Hettich, Mikro 22

Electrophoretic Equipment : Bio-Rad Sub Cell, GT

Magnetic Stirrers : Chiltern Hotplate Magnetic Stirrer

Power supplies : Bio-Rad Power PAC-300

Refrigerator : Philips, +4°C, -20°C

Transilluminator : Bio-Rad GelDoc 2000

Vortex : IKA LABORTECHNIK

Water Purification System : Millipore, Water Purification System.

Pipets :NichipetEX

Tips : Neptune BT brand barrier tips

Spectrophotometer : NanoDrop2000

3.1.3 Chemicals

Many of the chemicals were supplied from either MERCK (Germany) or SIGMA (USA). Alcohols were purchased from RIEDEL DE-HAEN (Germany).

3.1.4 Buffers and Solutions

During DNA isolation and visualization of the PCR products, different buffers were used.

- **DNA Isolation:** DNA was extracted from 200 mg of autoclaved meats and commercial food and feed samples following manufacturer's instructions using the Nucleospin Tissue Kit (Macherey–Nagel) and Qiagen DNeasy® Blood & Tissue Kit (QIAGEN, Germany).
- **PCR:** One buffer (Taq polymerase buffer (Takara, Biogen): 10x Taq Buffer +$[NH_4]_2SO_4$ – $MgCl_2$ (Fermentas, Germany) for Normal PCR and real-time PCR and master mix solution (SYBR® Green PCR Master Mix and FIREPol EvaGreen® qPCR Mix Plus (ROX) 1ml 08-24-00001 Solis Bio Dyne were used during the study.

- **Gel Electrophoresis:** Agarose gel electrophoresis was performed for visualization of PCR products. The buffers and solutions for the method were given in the list below.

  - ✓ 10 x TBE buffer: 54 g Trisbase, 27.50 g Boric acid, 4.650 g NaEDTA dissolved in 500 ml distilled water.
  - ✓ 10 x loading buffer : 2.5mg/ml bromophenol blue, 1% SDS in 2 ml of Glycerol
  - ✓ Ethididum Bromide : 10 mg/ml (Merck, Germany)
  - ✓ 2% agarose: 0.6 gram agarose (Merck, Germany) was dissolved in 30 ml boiling 0.5xTEB buffer.

3.1.5 Oligonucleotides Primers

Oligonucleotide primers were used to amplify specific gene loci, which are called 16S rRNA, 12S rRNA and Cyt b which were selected from published papers. Then, we checked specificity of DNAs in the DNA databank by using NCBI (National Center for Biotechnology Information) and BLAST programs (Table 3.2). All the primers were synthesized by the company, Metabion, Germany.

Table 3.2 Design of Oligonucleotide Primers of the various Plant and Animals Species

Primers	**Species**	**Genes**	**Positions**	**Oligonucleotides primers**	**Amplicons (bp)**
Horse	Equus caballus	Cyt b	DQ297663	5’ CCA GAA TGG TAC TTC CTG TTT GC 3’ 5’ TAG AGA GGA TTA GGG CTA ATA CGC 3’	85
Cow	*Bos taurus*	Cyt b	*Bos taurus* HQ 184045	5’ CAA GAA CAC TAA TGA CTA ACA TTC GAA AG 3’ 5’ AAA TGT TTG ATG GGG CTG GA 3’	93
Cow	*Bos taurus*	16S rRNA	*Bos taurus* EH 170825	5’ GTA GGT GCA CAG TAC GTT CTG AAG 3’ 5’ GGC CAG ACT GGG CAC ATG 3’	96
Soybean	Glycine max	lectin gene	Glycine max NC_016089	5’ CTTCTTTCTCGCACCAAT 3’ 5’ CTCAACAGCGACGACTTG 3’	100
Sheep	*Ovis aries*	12S rRNA	*Ovis Aries* NC 001941	5’ GAA AAA CCA TCG TTG TCA TTC AAC T 3’ 5’ AAA TAT TTG ATG GAG CTG GGA GA 3’	119
Goat	*Capra Hircus*	12S rRNA	*Capra Hircus* M 55541	5’ CTA GAG GAG CCT GTT CTA TAA TCG ATA A 3’ 5’ TGA CCT AAC GTC TTT ATG TGT GGT G 3’	142
Poultry	Gallus gallus	12S rRNA	*Gallus gallus* bNC 001323	5’ TGA GAA CTA CGA GCA CAA AC 3’ 5‘ GGG CTA TTG AGC TCA CTG TT 3’	183
Pork	Sus scrofa	Mt. DNA	AF039170	5’ GCCTAAATCTCCCCTCAATGGTA 3’ 5’ ATGAAAGAGGCAAATAGATTTTCG 3’	212
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

Table 3.2 (Cont.)

Beef	*Bos taurus*	Bovine ATPase8 subunit	NC_006853	5’ GCC ATA TAC TCT CCT TGG TGA CA 3’ 5‘ GTA GGC TTG GGA ATA GTA CGA 3’	271
Cow	*Bos taurus*	Mt. Cyt b	NC_006853	5’ GAC CTT CCA GCC CCA TCG AAC ATTTCA TCA TGA TGG AA 3’ 5’ CTA GAA AAG TGTA AGA CCC GTA ATA TAA G 3’	274
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
Ruminant	*Bos taurus*	16SrRNA-tRNA	*Bos taurus* EU177870	5’ GAA AGG ACA AGA GAA ATA AGG 3’ 5’ TAG CGG GTC GTA GTG GTT CT 3’	374

3.2 METHODS

3.2.1 Selection and Preparation of feed and food Samples

(a) To validate the commercial feed samples such as ruminants feed, the two grams raw meat of each species (cow, goat, sheep, pork, horse, fish, poultry and soybean) were autoclaved according to the European legislation (133°C at 300 kPa for 20 min). In order to evaluate the test sensitivity, a compound DNAs of all species (cow, goat, sheep, pork, horse, fish, poultry and soybean) was diluted in maize DNA up to 0.01%. Finally, known amounts (100%, 25%, 10%, 5%, 1%, and 0.1%) of (cow, goat, sheep, pork, horse, fish, poultry and soybean heat treated meats were minced and diluted in maize meal under controlled conditions.

b. To check the sensitivity of the assays, food samples were prepared by Vahdet Meat and Meat Products Endustri (Danet, Afyon, Turkey) as in Table 3.3.

Table 3.3 Preparation of samples for sensitivity.

	Beef/pork (%)	Soybean/poultry (%)	[k]Others (%)
1	60	20	20
2	30	10	60
3	3	1	96
4	0,3	0,1	99,6
5	0,03	0,01	99,96
6	0.003	0.001	99.99

[k]:Others: poultry/beef, spices, pepper.

The raw meats of various plant and animal species, commercial ruminants feed, pet foods, sausages, meat ball and salami were obtained from local supermarkets in Istanbul, Turkey. The raw meats were directly transported to the Genetic Research Laboratory of Fatih University and stored at -20°C until autoclaved and used for the extraction of the DNA in order to prevent the enzymatic degradation of DNA.

3.2.2 DNA Extraction, Quality and Quantity of DNA, Primer Design

i. DNA extraction:

DNA was extracted from 200 mg of food and feed samples using instructions and protocol of Nucleospin Tissue Kit (Macherey–Nagel) and Qiagen DNeasy® Blood & Tissue Kit (QIAGEN, Germany).

a. Principle/Procedure of NucleoSpin Tissue kit

NucleoSpin® Tissue is designed for the rapid purification of highly pure genomic DNA from tissue samples, mouse tails, bacteria, yeast, forensic samples (hair, dried blood spots, buccal swabs, cigarette filters), and clinical samples (stool, urine). Up to 35 µg of high-purity genomic DNA can be prepared (typical yields from tissue or cells: 15–25 µg). The obtained DNA can be used directly for PCR, Southern blotting,or any kind of enzymatic reaction. With the NucleoSpin® Tissue method, lysis is achieved by incubation of the samples in a solution containing SDS and Proteinase K at 56 °C. Appropriate conditions for binding of DNA to the silica membrane of the NucleoSpin® Tissue Columns are created by addition of large amounts of chaotropic ions (Binding Buffer B3) and ethanol to the lysate. The binding process is reversible and specific to nucleic acids. Contaminations are removed by efficient washing with buffer. Pure genomic DNA is finally eluted under low ionic strength conditions in a slightly alkaline elution buffer and is ready to use for subsequent reactions.

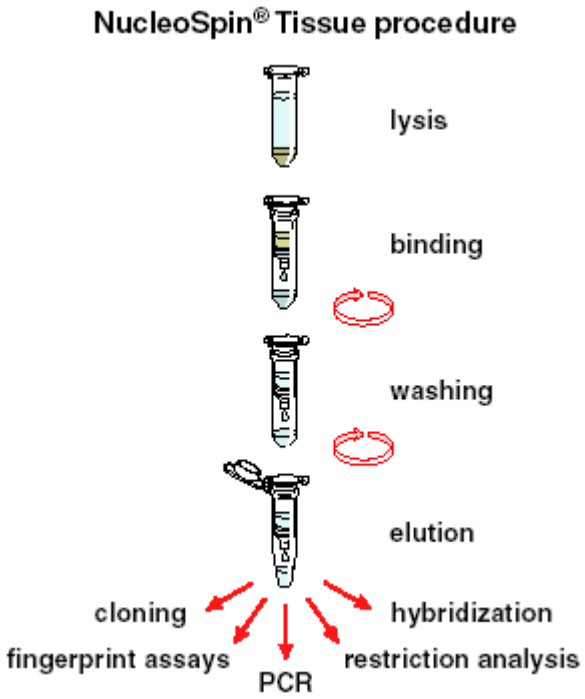


Figure 3.1 Principle/Procedure of NucleoSpin Tissue kit.

Protocol of Qiagen DNeasy® Blood & Tissue Kit

- 180 µl ATL buffer and 20 µl Proteinase K were mixed 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 AL buffer was added to the sample, vortexed thoroughly.
- 200 µl ethanol (96–100%) was added to mixture and vortexes to yield a homogenous solution.
- The homogenous solution was pipetted 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 tube was discarded and the DNeasy® mini column was put in a new 2 ml collection tube.
- 500 µl AW1 buffer was added and spun at 8000 RPM for 1 min.
- The flow-through and collection tube was discarded and the DNeasy® mini column was placed in another 2 ml collection tube.

- 500 µl 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 was removed.
- The DNeasy® mini column was placed in a clean 1.5 ml microcentrifuge tube. 200 µl AE buffer was pipetted directly onto the DNeasy® membrane and incubated at room temperature at 1 min.
- This was then spun at 8000 RPM for 1 min to elute. Elution was repeated to increase final DNA concentration. Last solution volume is 400 microliter in a microcentrifuge tube.
- After the amount of DNA was measured by spectrophotometer the DNA solution was stored at -20^{o}C until used in processes of PCR.

ii. Quantificaion of Nucleic Acids:

A spectrophotometer device (Nanodrop2000) was used to determine the concentration (quantification) of DNA in a solution. Samples were exposed to ultraviolet light at 260 nm and 280 nm. With the 260:280 ratio was determined qualification of nucleic acids. DNA concentration was approximately measured between 90 to 110ng and purity was between 1.85 to 200.

2.2.3 Polymerase Chain Reaction (PCR) Techniques

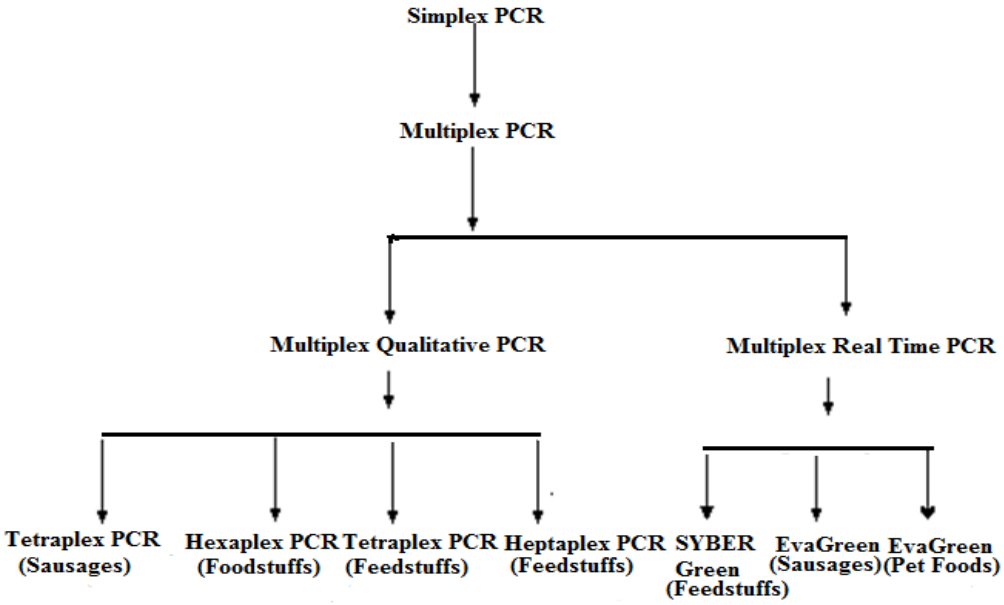


Figure 3.2 Modern PCR techniques which were developed by me in this thesis.

3.2.4 Polymerase Chain Reaction (PCR) Conditions

3.2.4.1 Simplex Qualitative PCR

PCR amplification was performed in a final volume of 25 µl containing EvaGreen dye, 0.1 mM of each primers and 70-90ng/µl of DNA template. Amplification was performed in a Thermocycler Techne with the following cycling conditions; after an initial heat denaturation step at 94^{o}C for 10 min, 35 cycles were programmed as follows: 94^{o}C for 30 s, 60^{o}C for 1 min, 72^{o}C for 1 min and final extension at 72^{o}C for 5 min.

Table 3.4 Simplex Qualitative PCR composition.

	PCR composition	Volume	Concentration
Master Mix	ddH_2O	17 µl	_
	Buffer	2 µl	1x
	Dntp	2 µl	0.2mM
	$MgCl_2$	2 µl	2mM
	Forward Primer	0.4µl	0.1mM
	Reverse Primer	0.4µl	0.1mM
	Taq polymerase	0.2µl	1U/reaction
	Template DNA	1 µl	70-90ng/µl
Total		25 µl	

3.2.4.2 Multiplex Qualitative PCR

For the simultaneous detection of each species, a one-step multiplex PCR was developed using each of the primer sets p reviously selected for the simplex PCR.

Table 3.5 Multiplex Qualitative PCR composition.

	PCR composition	Volume	Concentration
Master Mix	ddH_2O	17-10 µl	_
	Buffer	2 µl	1x
	dNTP	2 µl	0.2mM
	$MgCl_2$	2 µl	2mM
	Forward Primer	0.4µl for each species	0.1mM
	Reverse Primer	0.4µl for each species	0.1mM
	Taq polymerase	0.2µl	1U/reaction
	Template DNA	1 µl for each species	70-90ng/µl
Total		25 µl	

3.2.4.3 Agarose Gel Electrophoresis

A 2% agarose gel was prepared for the detection of the PCR products.

Preparation of the gel:

1. 0,6 g of agarose (Sigma, St. Louis, USA) was added to a 30 ml of 0.5 M Tris-Borate EDTA (TBE) buffer.
2. The mixture was boiled.
3. The gel was cooled to 60^{o}C and a 2 µl of ethidium bromide was added. It was poured to its plate and then a comb was placed into the gel.

Loading the samples: 2 µl loading dye +10 µl of PCR products + 100 bp DNA Ladder.+the gel was run at 110V in 0.5 M TBE buffer for 50 minutes.
0.5$_{X}$ TBE buffer was diluted from 10$_{X}$ TBE. To prepare 10$_{X}$ TBE buffer, 54 g Tris base, 27.50 g Boric acid, 4.650 g NaEDTA was dissolved in 500 ml distilled water.

3.2.5 Real-time PCR

Real-time PCR amplification was performed in a final volume of 20 µl containing SYBR Green master premix (Takara, Japan) and EvaGreen master mix, 0.1 Mm each of primers and 70-90ng/µl of DNA template (see Table 8). Amplification was performed in a Corbet Rotor-Gene 6000 rotary analyzer (Corbett, Australia) with the following cycling conditions; after an initial heat denaturation step at 94°C for 15 min, 40 cycles were programmed as follows: 94°C for 15 s, 60^{o}C for 30s, 72^{o}C for 30 s and after that melting curve analysis was programmed its ramp was formed from 72^{o}C to 95^{o}C raising by 1 C^{o} each step. Program waits for 118 s of pre-melt conditioning on first step and for 5 s for each step afterwards.

Table 3.6 Real-time PCR composition.

	PCR composition	**Volume**	**Concentration**
Master Mix	ddH$_2$O	13.2µl or 12.2µl	_
	Forward Primer	0.4µl	0.1mM
	Reverse Primer	0.4µl	0.1mM
	SYBR premix ex tag	4µl	1x
	Template DNA	2µl or 3µl	70-90ng/µl
	Total	20 µl	

3.2.5.1 Identification through Melting Curve Analysis

Melting Curve Analysis Tools of Rotor Gene Software Program (Corbett, Australia) at the end of each reaction was used to recognize specific-species melting temperature (tm) value of the amplified region of the template DNA.

3.2.6 RT-PCR's Sensitivity and Specificity

Progressive dilution of a multiple DNA template was diluted in soybean or maize DNA according to 10 fold: 1, 1/4, 1/16, 1/64, 1/128, 1/256, 1/1.000 dilutions. The results obtained from these dilutions at the end of PCR were shown both in raw data and melting curve analysis of software which is called Rotor-Gene. The detection limits were determined for cow, sheep and goat. Real-time dilution products also were run on a 2% agarose gel, and stained with ethidium bromide.

3.2.7 Multiplex Real-time PCR using SYBR green/EvaGreen dye (SMRT/EMRT-PCR)

Multiplex real-time PCR was applied to identify cow, soybean, pork and poultry etc materials in the same reaction with different assays. For this purpose different PCR conditions were used such as thermal gradients and amplification curves were compared with DNA bands in the results Qualitative multiplex PCR.

CHAPTER 4

RESULTS

4.1 OPTIMIZATION OF SIMPLEX PCR

A PCR assay, based on specific amplification of mt DNA with species-specific primers has been chosen for detection of adulteration horse, cow, soybean, sheep, goat, poultry, pork in foodstuff and feedstuffs. Simplex PCRs were optimized and carried out on DNA samples extracted from autoclaved meats to verify the specificity and sensitivity of the primers in the beginning PCR experiments. The primers generated specific fragments of 85bp, 93/96/271/274/374bp, 100bp, 119bp, 142bp, 183bp, 111/212/290bp, 224bp for horse, cow, soybean, sheep, goat, poultry, pork and fish species, respectively (see Figure. 4.1). To detect possible cross-reactions, each set of primers was performed in simplex PCR with non-target species and no false positive amplification was observed in related species.

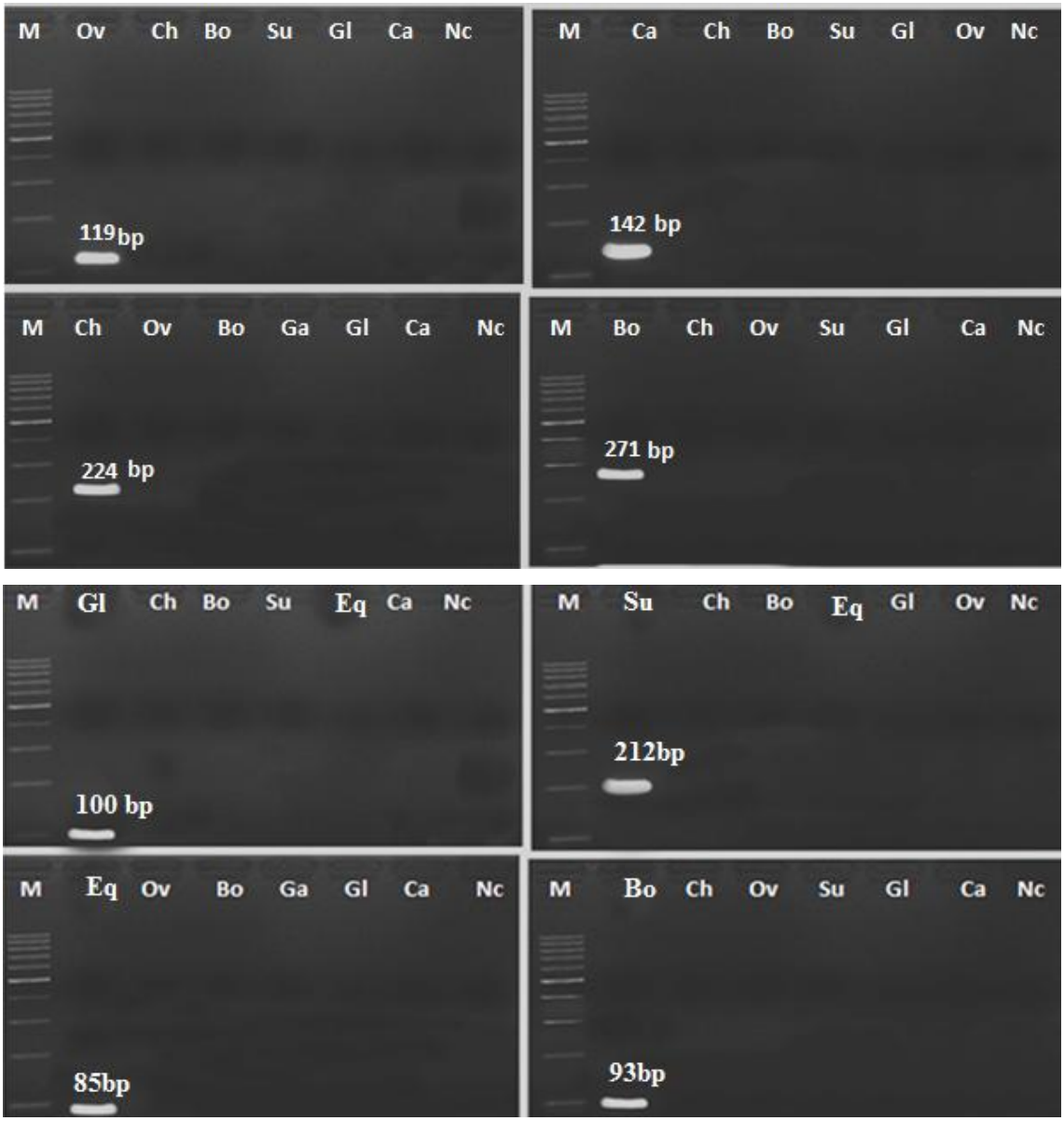


Figure 4.1 Specificity of primers of different species with pure relevant species samples. M: MARKER 100-bp, Ov: Ovis Aries (119bp), Ca: Capra hircus (142bp), Ch: Chrysophrys auratus (224bp), Bo: Bos Taurus (93bp, 271bp, 274bp, 374bp), Su: Sus scrofa (212bp, 290bp), Gl: Glycine max (100bp), Ga: Gallus gallus (183bp), Eq: Equus caballus (85), Nc: Negative control (reagents with primers without DNA).

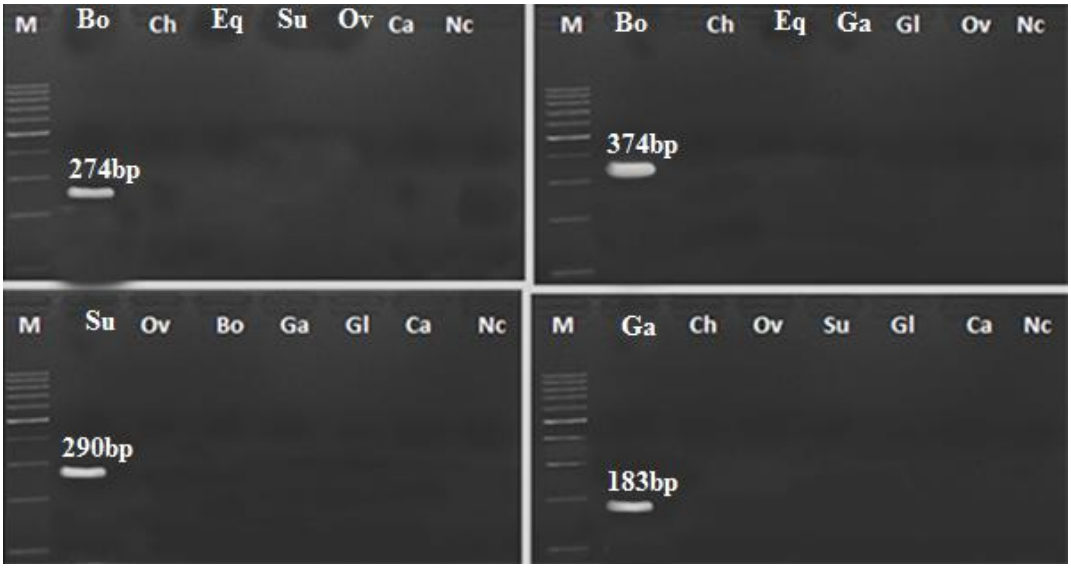


Figure 4.1 (Cont.)

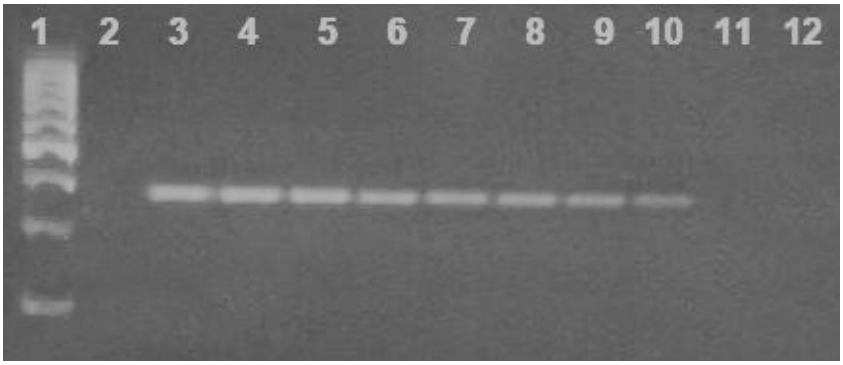


Figure 4.2 Progressive dilution of a multiple DNA template was diluted in soybean or maize DNA according to 10 fold of cow DNA template in pork, poultry and soybean: (1), M, 100-bp Marker; (2), control reagent; (3) positive control, (4) 1, (5) 1/4, (6) 1/16, (7) 1/64, (8) 1/128, (9) 1/256, (10) 1/1.000 dilutions.

4.2 TETRAPLEX (MULTIPLEX) PCR

4.2.1 Simplex and Tetraplex PCR Specificity and Optimization

Initially simplex and tetraplex PCRs were verified by the DNAs extracted from heat-treated ovine, caprine, bovine and fish meats (see Figure 4.3).

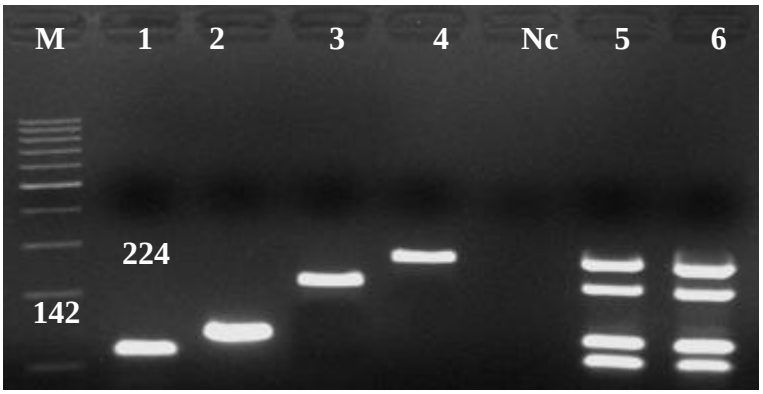


Figure 4.3 Specificity of simplex and tetraplex assay of DNA from autoclaved meats: M: Marker, 100 bp, (1) Ovis aries, (2) Capra hircus, (3) Chrysophrys auratus, (4) Bos taurus, (Nc) Negative control (reagents with primers but no DNAs), (5) Tetraplex (Ovis aries, Capra hircus, Chrysophrys auratus, Bos taurus), (6) Tetraplex (Ovis aries, Capra hircus, Chrysophrys auratus, Bos taurus) repeated.

4.2.2 Sensitivity test

The sensitivity of PCR targeting species DNAs were verified until the minimum amount of 0.01% tested in soybean DNA (see Figure 4.4). Moreover, reference samples sensitivity results showed that the sensitivity threshold was 0.1% (see Figure 4.5).

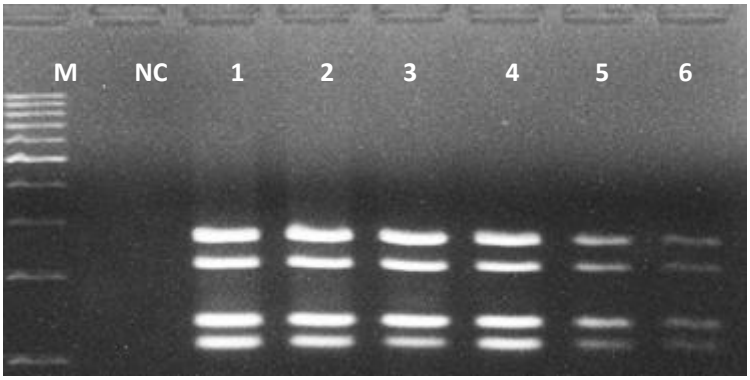


Figure 4.4 Evaluation of tetraplex assay DNA based sensitivity with mixed DNA template: progressive dilution of mixed DNAs template diluted in DNA of soybean. M: MRKER 100-bp. NC: Negative control (reagents with primers without DNAs) (1) 100%, (2) 25%, (3) 5%. (4) 1%, (5) 0.1%, (6) 0.01%.

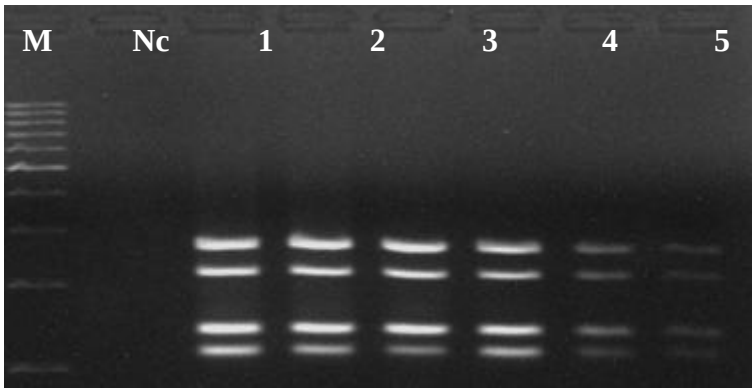


Figure 4.5 Evaluation of tetraplex assay sensitivity on mixed heat treated meats in soybean meal. M: MRKER 100-bp. Nc: Negative control (reagents with primers without DNAs) (1) 100%, (2) 25%, (3) 10%, (4) 5%, (5) 1%, (6) 0.1%

4.2.3 Application of Tetraplex PCR assay on Feedstuffs

The application of the assays to commercial feeds and pet foods has been demonstrated in Figure 4.6 (A, B, C), and Table 4.1 which reports the true species composition of the listed sample products.

The results of Tetraplex assays showed that fish origin pet food contained the same contents as labeled and had no contamination. However, the lamb pet food contained the other species (Fish, goat and bovine) with labeled. Similarly, beef pet food was also contaminated with sheep and fish origin contents. The results of ruminants feed tested samples showed that 60% samples were contaminated with animal origin contents.

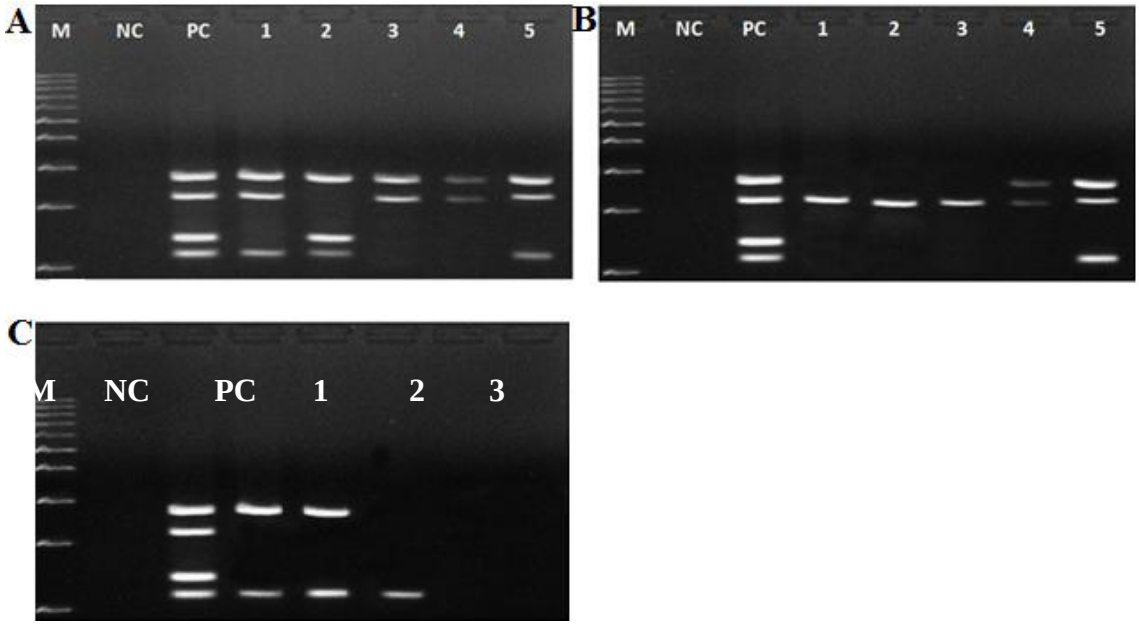


Figure 4.6 Tetraplex PCR on pet food and ruminants feed. M: Marker 100-bp, NC: negative control (reagents with primers without DNAs), PC: positive control; (A) lane1-5, pet food, (B) lane1-5, pet food (C) lane1-5, ruminants feed.

Table 4.1 Results of Tetraplex PCR performed on commercial pet food and ruminant feed.

Products	Labeled	Results
Pet foods, n® : 4	Lamb	Sheep, goat, fish and cow
Pet foods, n® : 3	Fish	Fish
Pet foods, n® : 3	Beef	Sheep, fish and cow
Ruminants feed, n®:5	nat	Cow and sheep

nat: no animal tissues in these samples

n® : number of products

4.3 HEPTAPLEX (MULTIPLEX) PCR

4.3.1 Simplex and Heptaplex PCR Specificity and Optimization

Initially simplex and Heptaplex PCRs were verified by the DNAs extracted from heat-treated horse, soybean, pork, poultry, ovine, bovine and fish meats (Figure 4.7).

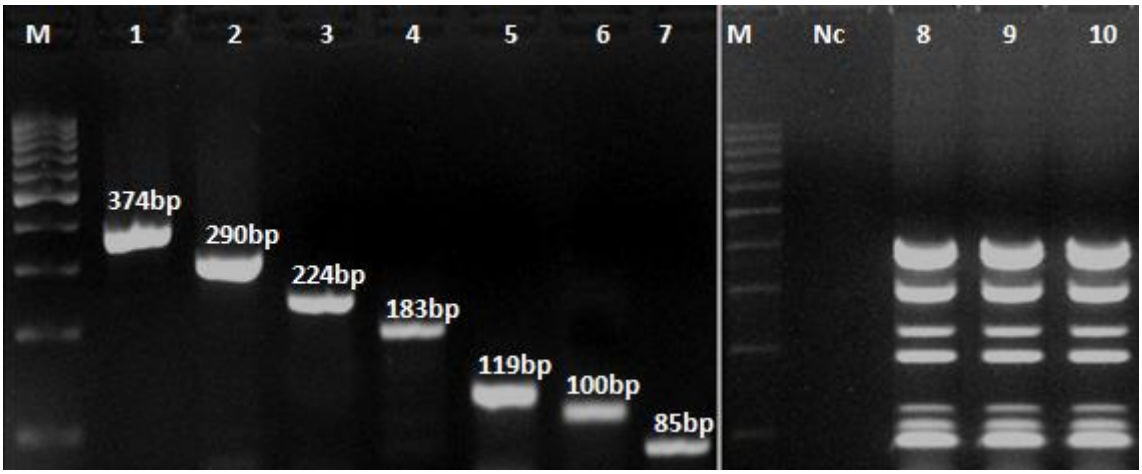


Figure 4.7 Specificity of simplex and Heptaplex assay of DNA from autoclaved meats: M: Marker, 100 bp, (1) Bos taurus, (2) Sus Scrofa, (3) Chrysophrys auratus, (4) Bos taurus, (4) Gallus gallus (5) Ovis aries, (6) Glycine max, (7) Equus caballus (Nc) Negative control (reagents with primers but no DNAs), (8,9,10 repeat) Heptaplex (Bos taurus, Sus Scrofa, Chrysophrys auratus, Gallus gallus, Ovis aries, Glycine max, Equus caballus).

3.3.2 Sensitivity test

The sensitivity of PCR targeting species reference samples DNAs were verified until the minimum amount of 0.01% tested in maize DNA (see Figure 4.8).

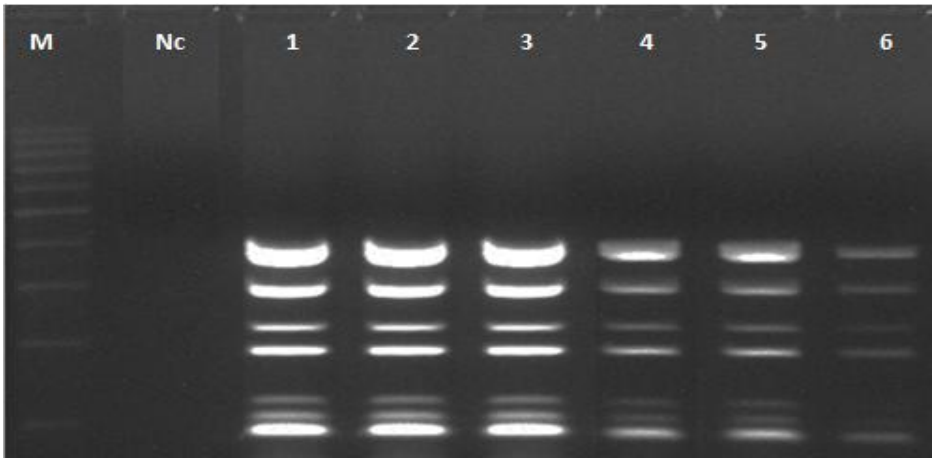


Figure 4.8 Evaluation of Heptaplex assay sensitivity on mixed heat treated meats in maize meal. M: MRKER 100-bp. NC: Negative control (reagents with primers without DNAs) (1) 30%, (2) 15%, (3) 5%, (4) 1%, (5) 0.1%, (6) 0.01%

4.3.3 Application of Heptaplex PCR assays on Feedstuffs

The application of the assays to commercial pet foods, animal meals, ruminants' feeds and baby foods has been verified in Figures 4.9 (a, b, c, d) and Table 4.2 which reports the true species composition of the listed sample products.

The results of Heptaplex PCR assays showed that fish and poultry origin pet food contained the same contents as labeled and had no contamination. The animal meals results showed that bovine meat and bone meal was contaminated with poultry, sheep and horse contents as labeled. Soybean meal was contaminated with sheep contents. Poultry meal contained the other species (Fish, sheep and horse) as written on label. Similarly, the results of ruminants feed tested samples showed that they were contaminated with poultry and lamb origin contents as labeled. However, baby foods contained the same contents as labeled and had no contamination.

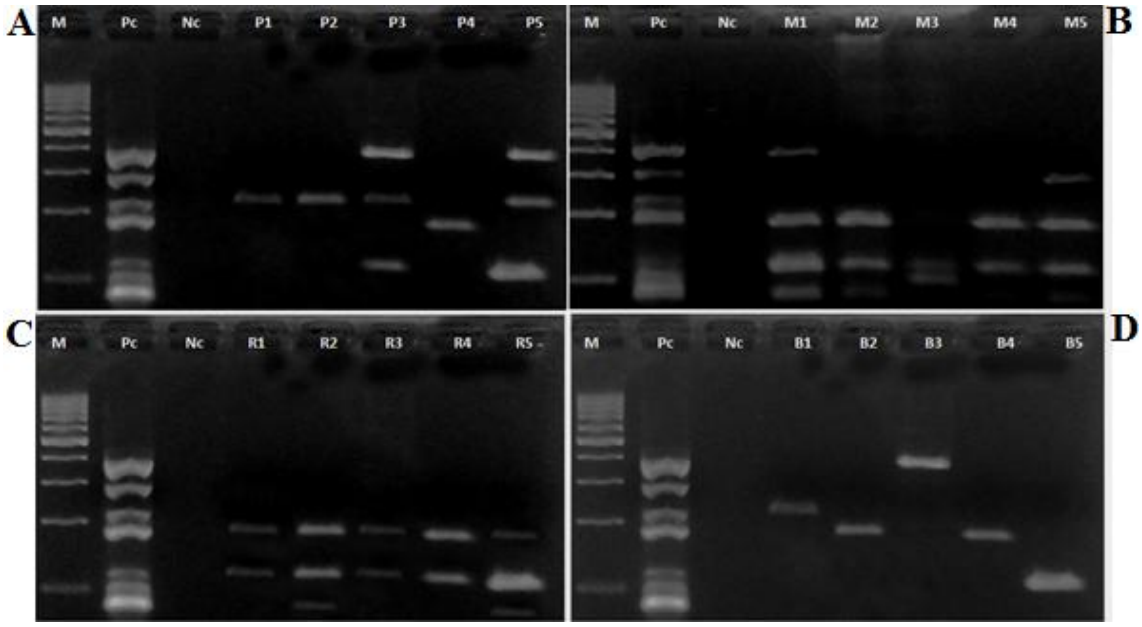


Figure 4.9 Application of Heptaplex PCR on pet food, baby foods, ruminants' feed and animal meals. M: Marker 100-bp, Nc: negative control (reagents with primers without DNAs), Pc: positive control; (a) P1-P5: pet foods (b) M1-M5: animal meals (c) R1-R5: ruminants feeds (d) B1-B5: baby foods.

Table 4.2 Results of Heptaplex PCR performed on Commercial Pet Food, Animal meal, Ruminant Feed and Baby Food.

Products	Labeled	Results
Pet food, n® : 2	Fish	Fish
Pet food, n® : 1	Poultry	Poultry
Pet food, n® : 2	Lamb	Sheep, fish and cow
Animal meal, n® : 1	Beef	Bovine, sheep, poultry, horse
Animal meal, n® : 3	Poultry	Poultry, fish, sheep, horse
Plant meal, n® : 1	Soybean	Sheep
Ruminants feed, n®: 5	Nat	Poultry, sheep
Baby food, n®: 1	Beef	Beef
Baby food, n®: 2	Poultry	Poultry
Baby food, n®: 1	Fish	Fish
Baby food, n®: 1	Lamb	lamb

Nat : no animal tissues in these samples

n® : number of products

4.4 TETRAPLEX (MULTIPLEX) PCR

4.4.1 Simplex and Tetraplex PCR Specificity and Optimization

In an elementary phase of this research, simplex and tetraplex PCRs were verified by the DNAs extracted from processed soybean, poultry, horse, and pork meats. The amplification of total DNAs of reference sausages yielded the PCR fragments of 100 bp, 183 bp, 85 bp and 212 bp for soybean, poultry, horse, and pork species, respectively (see Figure 4.10).

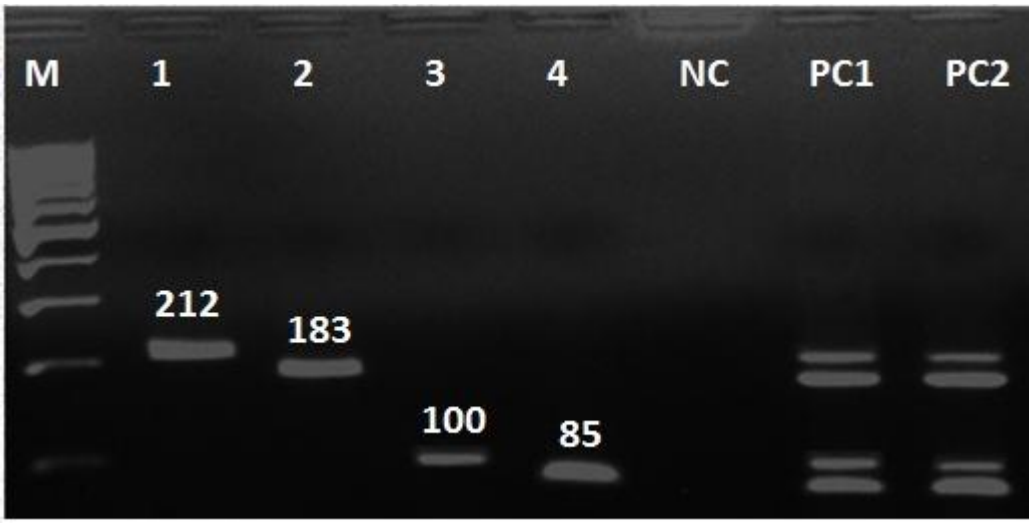


Figure 4.10 Specificity of simplex and Tetraplex assay of DNA from processed sausages: M: Marker, 100 bp, (1) Sus scrofa, (2) Gallus gallus, (3) Glycine max, (4) Equus caballus (NC) Negative control (reagents with primers but no DNAs), (PC1, PC2) Tetraplex of Sus scrofa, Gallus gallus, Glycine max, Equus caballus.

4.4.2 Sensitivity Test

Tetraplex PCR assays were carried out for soybean, poultry, horse, and pork species identification in processed sausages to evaluate sensitivity of the assay. Reference samples sensitivity results showed that the sensitivity threshold was 0.01% in beef DNAs (see Figure 4.11).

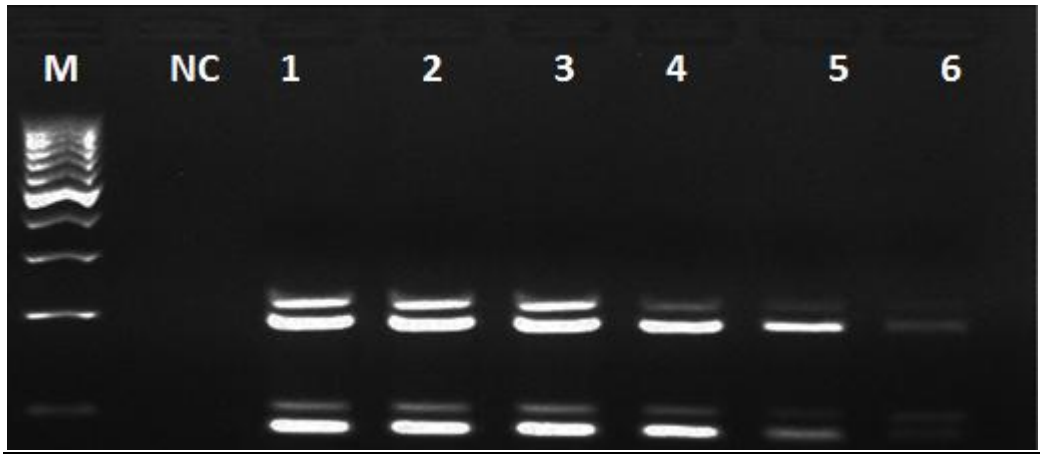


Figure 4.11 Evaluation of Tetraplex assay sensitivity for soybean, poultry, horse and pork from processed sausages; M: Marker 100-bp. NC: Negative control (reagents with primers without DNAs) (1) 100% (2) 20%, (3) 10%, (4) 1%, (5) 0.1%, (6) 0.01%.

4.4.3 Application of Tetraplex PCR assay on commercial sausages

The application of the assays to commercial sausages has been depicted in Figures 4.12 (A, B, C, D) and Table 4.3, which shows the accurate species composition of the submitted sausages sample. The results of Tetraplex assays showed that horse and pork origin samples contained the same contents as labeled and had no contamination. The results of beef sausages tested samples showed that 60% samples were contaminated with poultry origin while 40% samples were contaminated with soybean protein origin contents. Similarly, 60% beef and poultry mixed sausages samples were also contaminated with soybean protein origin contents. Finally, the results of beef, poultry and soybean mixed sausages tested samples showed that 100% samples were verified correctly as labeled.

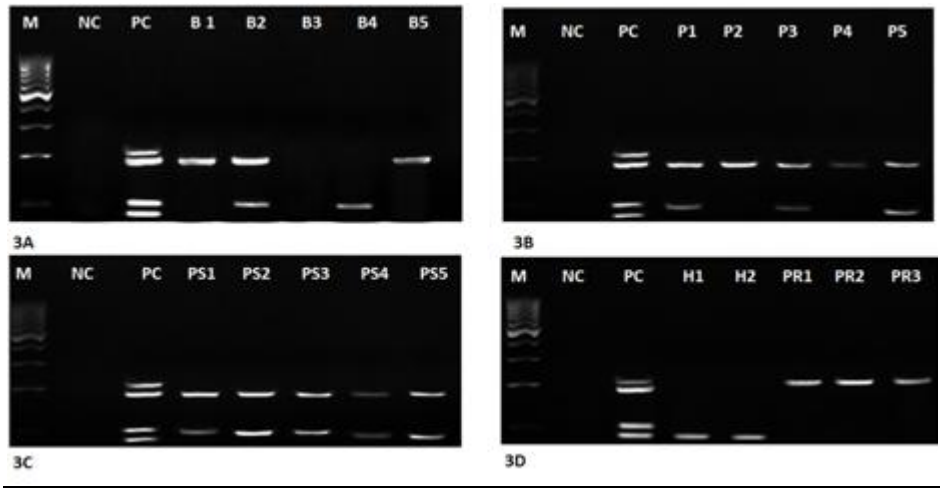


Figure 4.12 Applicability of Tetraplex PCR on commercial sausages. M: Marker 100-bp, NC: negative control (reagents with primers without DNAs), PC: positive control, Fig. 3A (B1-B5:100% beef samples), Fig. 3B (P1-P5:100% poultry samples), Fig. 3C (PS1-PS5:100% poultry and soya mixed samples), Fig. 3D (H1, H2:100% horse samples while PR1-PR3:100% pork samples).

Table 4.3 Results of Tetraplex PCR performed on commercial sausages.

Products	Labeled	Results
Sausages, n® : 5	Beef	Soybean, poultry
Sausages, n® : 5	Poultry	Soybean, poultry
Sausages, n® : 5	Beef+ poultry+ soybean	Soybean, poultry
Sausages, n®: 3	Pork	Pork
Sausages, n®: 2	Horse	Horse

n® : number of samples

4.5 HEXAPLEX (MULTIPLEX) PCR

4.5.1 Simplex and Hexaplex PCR Specificity and Optimization

Initially simplex and Hexaplex PCRs were verified by the DNAs extracted from heat-treated horse, soybean, pork, poultry, ovine, bovine and fish meats (Figure 4.13).

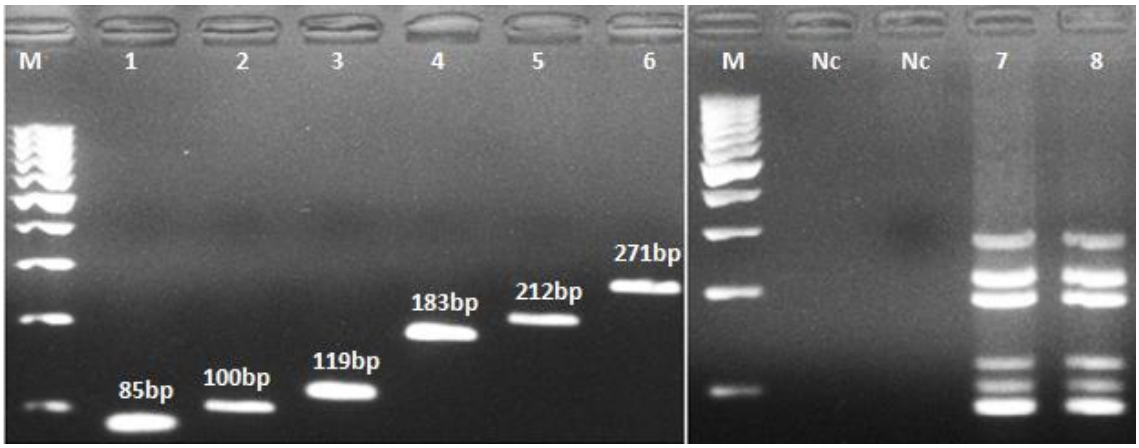


Figure 4.13 Specificity of simplex and Heptaplex assay of DNA from autoclaved meats: M: Marker, 100 bp, (1) Bos taurus, (2) Sus Scrofa, (3) Chrysophrys auratus, (4) Bos taurus, (4) Gallus gallus (5) Ovis aries, (6) Glycine max, (7) Equus caballus (Nc) Negative control (reagents with primers but no DNAs), (8,9,10 repeat) Heptaplex (Bos taurus, Sus Scrofa, Chrysophrys auratus, Gallus gallus, Ovis aries, Glycine max, Equus caballus).

4.5.2 Sensitivity test

Hexaplex PCR assays were carried out for bovine, sheep, soybean, poultry, horse, and pork species identification in processed sausages to evaluate sensitivity of the assay. Reference samples sensitivity results showed that the sensitivity threshold was 0.01%. (see Figure 4.14).

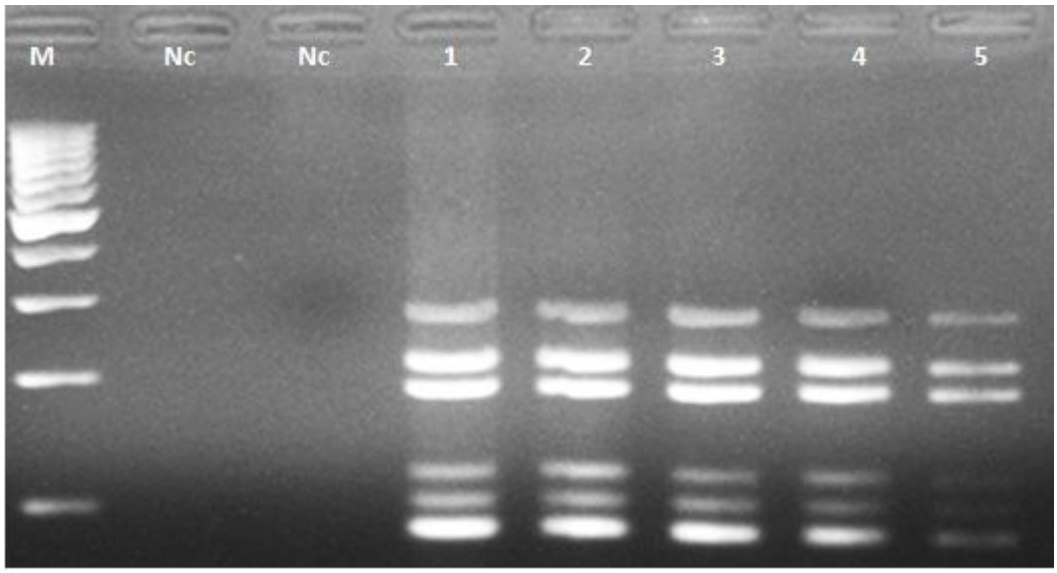


Figure 4.14 Evaluation of Hexaplex assay sensitivity for bovine, sheep, soybean, poultry, horse and pork from processed sausages; M: Marker 100-bp. NC: Negative control (reagents with primers without DNAs) (1) 30% (2) 20%, (3) 10%, (4) 1%, (5) 0.1%, (6) 0.01%.

4.5.3 Application of Hexaplex PCR assay on commercial sausages

The application of the assays to commercial sausages has been depicted in Figures 4.15 (A, B, C, D), and Table 4.4 which shows the correct species composition of the submitted sausage samples. The results of Hexaplex assays showed that horse and pork origin samples contained the same contents as labeled and had no contamination. But the results of lamb sausages were interesting as in 2/5 samples contained beef origin instead of lamb as labeled. The results of beef sausages tested samples showed that 60% samples were contaminated with poultry origin while 40% samples were contaminated with soybean protein origin contents. Similarly, 60% sausages samples contained beef and poultry were also contaminated soybean protein contents. The results of poultry and soybean mixed sausages were also confirmed except one sample as written on label. Finally, the results of beef, poultry and soybean mixed sausages tested samples showed that 100% samples were verified correctly as labeled.

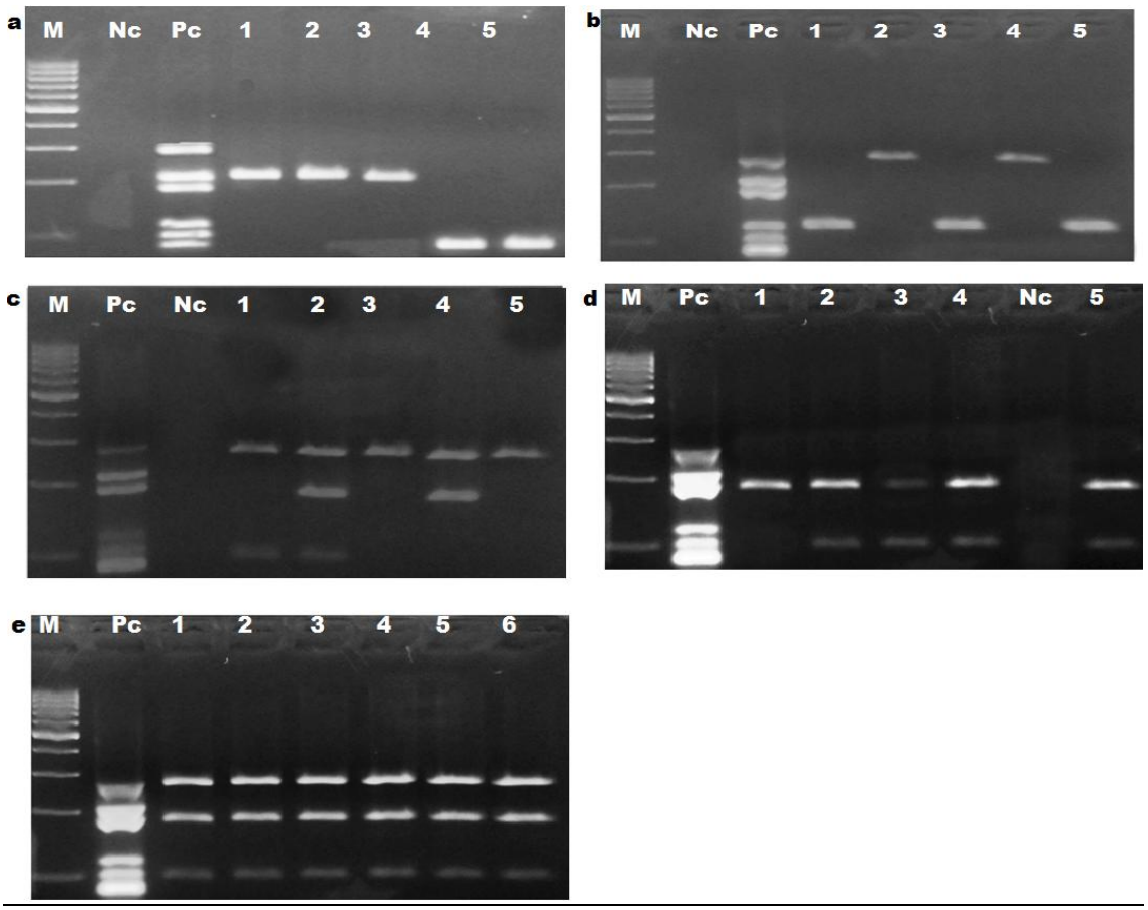


Figure 4.15 Applicability of Hexaplex PCR on commercial sausages. M: Marker 100-bp, Nc: negative control (reagents with primers without DNAs), Pc: positive control, (a) (1-3:100% pork and 4,5: 100% horse), (b) (1-5:100% lamb), (c) (1-5:100% beef), (d) (1-5: poultry and soya mixed) (e) (1-6: beef, poultry and soya mixed).

Table 4.4 Results of Hexaplex PCR performed on commercial sausages

Products	Labeled	Results
Sausages, n℗ : 3	Pork	Pork
Sausages, n℗ : 2	Horse	Horse
Sausages, n℗ : 5	Lamb	lamb, beef instead of lamb
Sausages, n℗ : 5	Beef	Beef, soybean, poultry
Sausages, n℗: 5	Poultry+ soybean	poultry, soybean
Sausages, n℗: 5	Beef + soybean +poultry	Beef, soybean, poultry

n℗ : number of samples

4.6 IDENTIFICATION OF SPECIES BY REAL TIME PCR MELTING CURVE ANALYSIS

Real time PCR technique was optimized. All of the reference strains (cow, sheep and goat) were amplified. Real-time results of PCR amplification products were shown using Rotor-Gene 6000 system employing EvaGreen and SYBR Green. All of the reference strains were amplified several times and melting curve chart belonging to cow, soybean, poultry and pork (see Figure 4.16) was obtained by using real-time PCR melting curve analysis program in the Rotor-Gene Software. Temperature values belonging to three species were identified according to melting curve peaks. It was seen that each species has a different characteristic Tm value. Test results were considered positive when their melting Tm was within the average Tm ± 0.4 for each class of species. By looking at the characteristic Tm value which is specific to the species, it can be easily identified that the sign as a result on the machine is a false positive primer-dimers or a true positive PCR product. Alternatively, one can easily say what the origin of the amplicons belongs to which one.

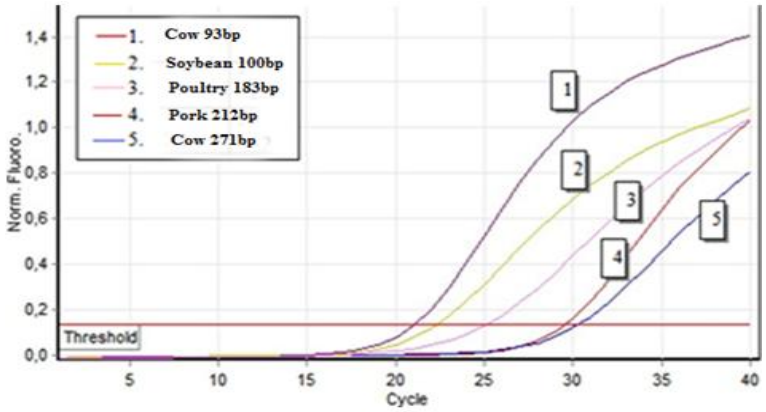


Figure 4.16 Quantification raw data chart of species. It shows raw data analysis of four different species. Curve 1 and 5, cow; curve 2; soybean, curve 3; poultry and curve 4, pork.

4.7 IDENTIFICATION OF REAL TIME PCR ASSAY SENSITIVITY

Progressive dilution of a poultry 50ng DNA template was diluted in soybean, pork and cow DNAs according to 10 fold: 10%, 1%, 0.1%, 0.01%, 0.001%, and

0.0001% dilutions. The results obtained from these dilutions at the end of PCR were shown in melting curve analysis of software which is called Rotor-Gene. The detection limits of poultry DNA template in pork, cow and soybean DNA were determined 0.0001% (see Figure 3.17).

a

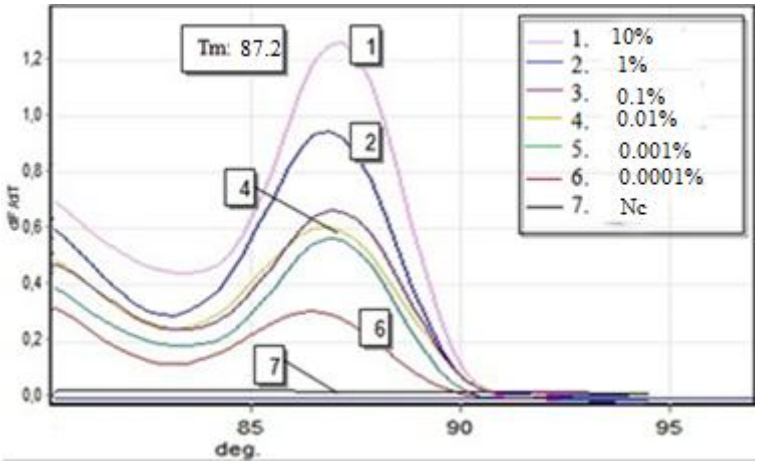


Figure 4.17 Melting curves of diluted poultry DNA (a) and gel analysis (b)

b

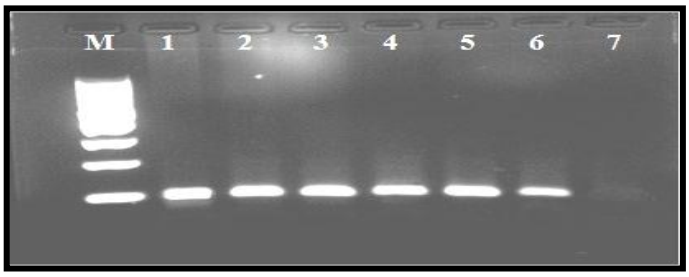


Figure 4.17 (conti.)

4.8 SYBER GREEN DUPLEX REAL TIME PCR (SDRT)

4.8.1 Development of SDRT-PCR

The primers for poultry and bovine were mixed for the multiplex reaction. The templates were amplified in the real-time PCR followed by a melting curve analysis using SYBR Green and the Corbett Rotor Gene Analyzer System. The addition of amplicons in the same reaction was verified in a graph representing the changes in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. Bovine and poultry amplicons were easily distinguished through specific tm values due to the different length and base compositions of two amplicons. The SDRT-PCR resulted in a single curve with two peaks as shown in Figure 4.18. These peaks formed at a specific location on the temperature axis at 79.5 °C for bovine and 87.2 °C for poultry.

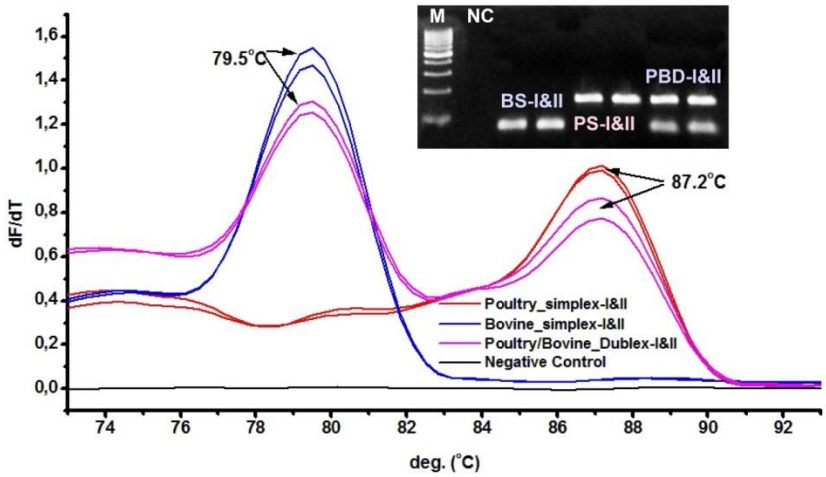


Figure 4.18 Specificity of the SDRT-PCR assay with fluorescence melting curve for bovine and poultry in SYBR Green duplex real-time PCR performed with Corbett Rotor Gene Analyzer and confirmation by gel electrophoresis. Melting temperature profiles of bovine and poultry: PS I &II (poultry183bp_simplex repeat positive control), tm: 87.2°C; BS I &II (bovine93bp_simplex repeat positive control), tm: 79.5°C; PBD I &II (poultry/bovine_duplex repeat positive control), tm: 87.2°C & 79.5°C; NC (negative control); M: 100 bp ladder.

4.8.2 Specificity of SDRT-PCR system

For the duplex determination of bovine and poultry tissue in feedstuffs, a primer specific to bovine was designed Lopez-Andreo et al. (2005), and a poultry primer was

designed by Dalmasso et al. (2004) were used. Both primers were used to develop amplification conditions. The specificity of the primers was tested for seven livestock species: pork, horse, soybean, bovine, sheep, chicken and turkey. It was verified that primers specific to the species of bovine and poultry showed no cross-reaction with any of the non-target species. Specificity of the duplex assay was developed by carrying out melting curve analysis. This duplex real-time PCR assay was specific for each species investigated with slightly varying melting temperatures (tm). The tm values of bovine and poultry genes were recorded as 79.5 ± 0.4 °C and 87.2 ± 0.3 °C. SDRT-PCR yields were run on a 2% agarose gel stained with ethidium bromide to crosscheck. An agarose gel electrophoresis of the PCR products showed that bovine and poultry samples produced clear bands of the expected size of 93 and 183 bp, respectively (see Figure 4.18).

4.8.3 Sensitivity of the SDRT-PCR system

To determine the sensitivity and linearity of the real-time PCR technique, the genomic DNA was obtained from each target species starting from the 45 ng target DNA. Experiments with samples of decreasing concentration indicated that bovine and poultry DNA concentrations as low as 0.0001 ng/ul still yielded an amplicons signal upon SDRT-PCR (Figure not shown). Amplification reactions with mixtures of bovine and poultry DNA samples further demonstrated that as little as 0.001% bovine and poultry DNA could be detected against a background of 45 ng poultry and 45 ng bovine DNA, respectively. SDRT-PCR yields were run on a 2% agarose gel to crosscheck and it confirmed the sensitivity of the assay (see Figure 4.19).

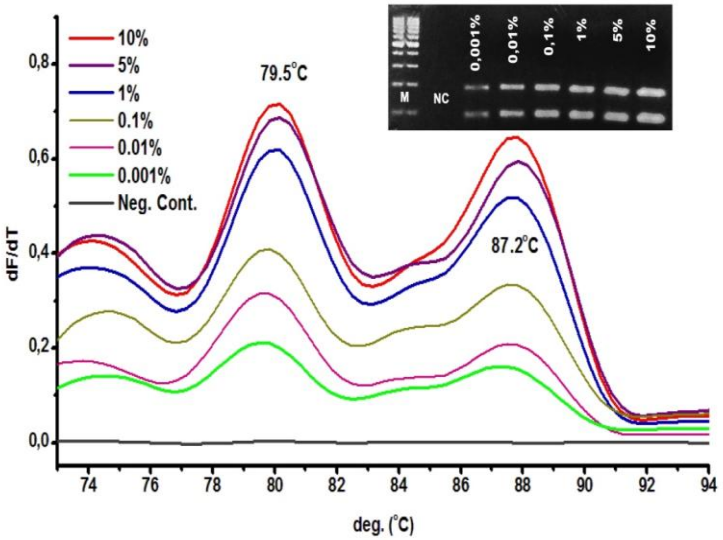


Figure 4.19 Determination of relative sensitivity by using known heat treated meat mixtures of bovine and poultry DNA in different ratios (10%, 5%, 1%, 0.1%, 0.01%, 0.001%). Peaks at tm: 87.2°C & 79.5°C correspond to amplicons obtained with poultry and bovine DNAs, respectively. NC: negative control. M: 100 bp ladder.

4.8.4 Application of the SDRT-PCR assays on commercial feedstuffs

The application of the SDRT-PCR assays to commercial animal feed products has been confirmed in Figures 4.20-4.22 and Table 4.5. With regard to commercial animal feeds, only in poultry feeds the species claimed in label has been confirmed by the assay analysis (see Figure 4.20).The results are shown in Figure 4.21 which indicated that 40% bovine samples were contaminated with poultry residuals, which were labeled as 100% beef. These residuals are not in accordance with the ingredients labeled by the producer. However, the animal feed samples which contain beef and poultry, claimed constituents have been confirmed by the SDRT-PCR analysis (see Figure 4.22). The 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 feed and food products to verify the origins of
raw material.

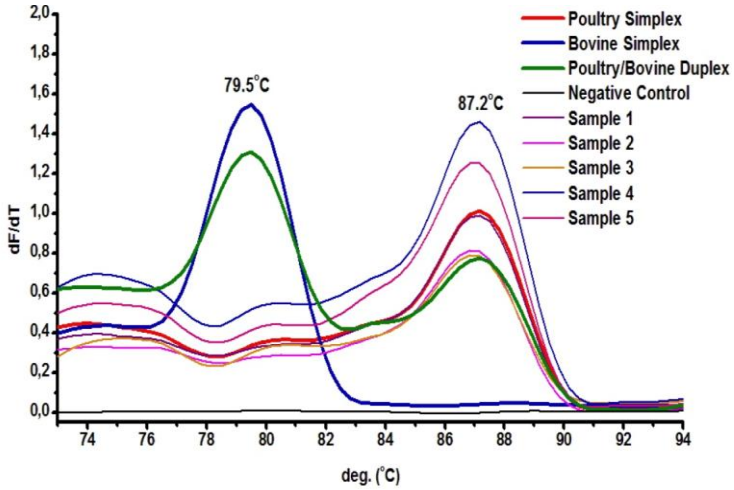


Figure 4.20 SDRT-PCR assay with DNA samples from feed contained 100% poultry using primer pairs specific to bovine and poultry. Peaks (tm: 79.5°C) correspond to amplicons obtained with DNA samples from feed contained beef. Peaks (tm: 87.2°C) correspond to amplicons obtained with DNA samples from poultry positive samples. Poultry (183bp) simplex (positive control), Bovine (93bp) simplex (positive control), Poultry and bovine duplex (positive control), NC: negative control.

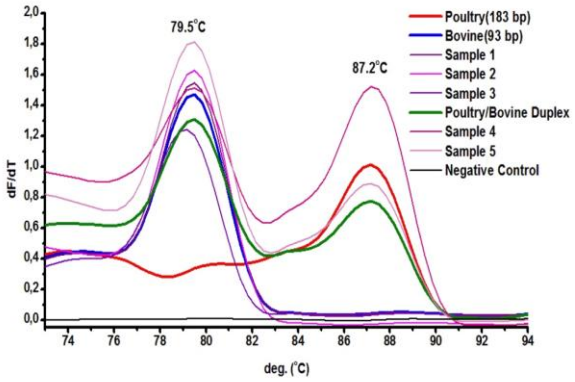


Figure 4.21 SDRT-PCR assay with DNA samples from feed contained 100% beef using primer pairs specific to bovine and poultry. Peaks (tm: 79.5°C) correspond to amplicons obtained with DNA samples from feed contained beef. Other peaks (tm: 87.2°C) correspond to amplicons obtained with DNA samples from poultry contamination. Poultry (183bp) simplex (positive control), Bovine (93bp) simplex (positive control), Poultry and bovine duplex (positive control), NC: negative control.

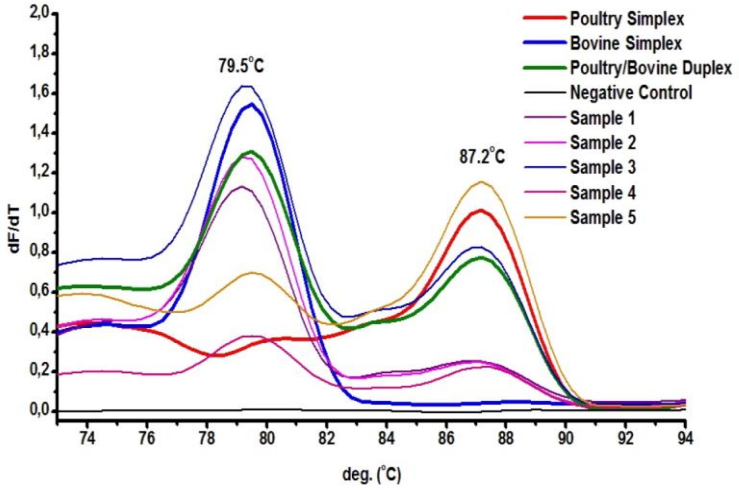


Figure 4.22 SDRT-PCR assay with DNA samples from mixed feed contained beef and poultry using primer pairs specific to bovine and poultry. Peaks (tm: 79.5°C) correspond to amplicons obtained with DNA samples from feed contained beef. Other peaks (tm: 87.2°C) correspond to amplicons obtained with DNA samples from poultry. Poultry (183bp) simplex (positive control), Bovine (93bp) simplex (positive control), Poultry and bovine duplex (positive control), NC: negative control.

Table 4.5 Results of SDRT-PCR performed on commercial feedstuffs

Products	Labeled	Results
Feedstuff, n® : 5	Poultry	poultry
Feedstuff, n® : 5	beef	beef, poultry
Feedstuff, n® : 5	beef+poultry	beef+poultry

n® : number of samples

4.9 EVAGREEN MULTIPLEX REAL TIME PCR (EMRT)

4.9.1 Development of EMRT-PCR

The primers for soybean and bovine were mixed for the multiplex reaction. The templates were amplified in the real-time PCR followed by a melting curve analysis using EvaGreen and the Corbett Rotor Gene Analyzer System. The addition of amplicons in the same reaction was verified in a graph representing the changes in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. Bovine and soybean amplicons were easily distinguished through specific tm values due to the different length and base compositions of two amplicons. The EMRT-

PCR resulted in a single curve with two peaks as shown in Figure 4.23. These peaks formed at a specific location on the temperature axis at 82 °C for bovine and 86 °C for bovine and soybean respectively.

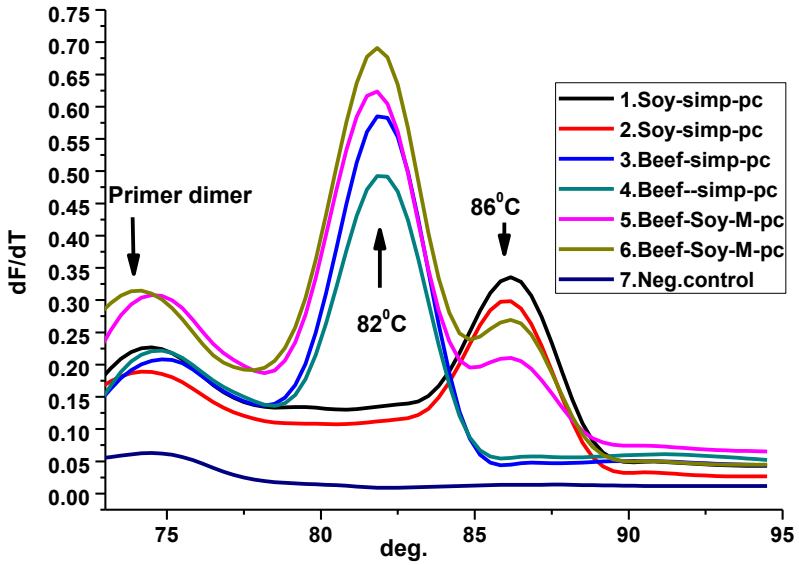


Figure 4.23 Specificity of the EMRT-PCR assay with fluorescence melting curve for beef and soybean in EvaGreen multiplex real-time PCR performed with Corbett Rotor Gene Analyzer. Melting temperature (tm) profiles of beef and soybean; Beef_simplex_ repeat positive control, tm: 82°C; Soybean_simplex_repeat positive control, tm: 86°C; Poultry/bovine_multiplex repeat positive control (multiplex amplification), tm: 82°C & 86°C; Negative control.

4.9.2 Specificity of EMRT-PCR system

For the multiplex determination of bovine and soybean tissue in reference and commercial sausages, a primer specific to bovine was designed Lahiff et al. (2001), and a soybean primer was designed by Zhang et al. (2007) were used. Both primers were used to develop amplification conditions. The specificity of the primers was tested for seven species: pork, horse, soybean, bovine, sheep, chicken and turkey. It was verified that primers specific to the species of bovine and soybean showed no cross-reaction with any of the non-target species. Specificity of the multiplex assay was developed by carrying out melting curve analysis. This multiplex real-time PCR assay was specific

for each species investigated with slightly varying melting temperatures (tm). The tm values of beef and soybean genes were recorded as 82 ± 0.2 °C and 86 ± 0.3 °C. EMRT-PCR yields were run on a 2% agarose gel stained with ethidium bromide to crosscheck. An agarose gel electrophoresis of the PCR products showed that beef and soybean samples produced clear bands of the expected size of 271 and 100 bp, respectively (see Figure 4.24).

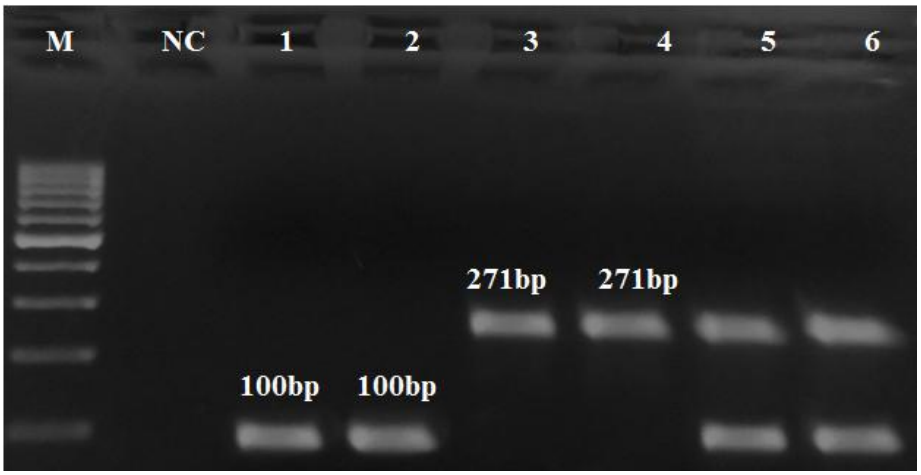


Figure 4.24 Agarose gel electrophoresis of EMRT-PCR products. M: 100 bp marker; NC: negative template control; 1&2: Soybean positive control; 3&4: bovine positive control; 5&6: soybean _ beef multiplex positive control.

4.9.3 Sensitivity of the EMRT-PCR system

To determine the sensitivity and linearity of the real-time PCR technique, the genomic DNA was obtained from each target species starting from the 45 ng target DNA. Experiments with samples of decreasing concentration indicated that beef and soybean DNA concentrations as low as 0.0001 ng/ul still yielded an amplicons signal upon EMRT-PCR (Figure not shown). Amplification reactions with mixtures of beef and soybean DNA samples further demonstrated that as little as 0.003% bovine and 0.001% soybean DNA could be detected (see Figure 4.25). EMRT-PCR yields were run on a 2% agarose gel to crosscheck and it confirmed the sensitivity of the assay (see Figure 3.26).

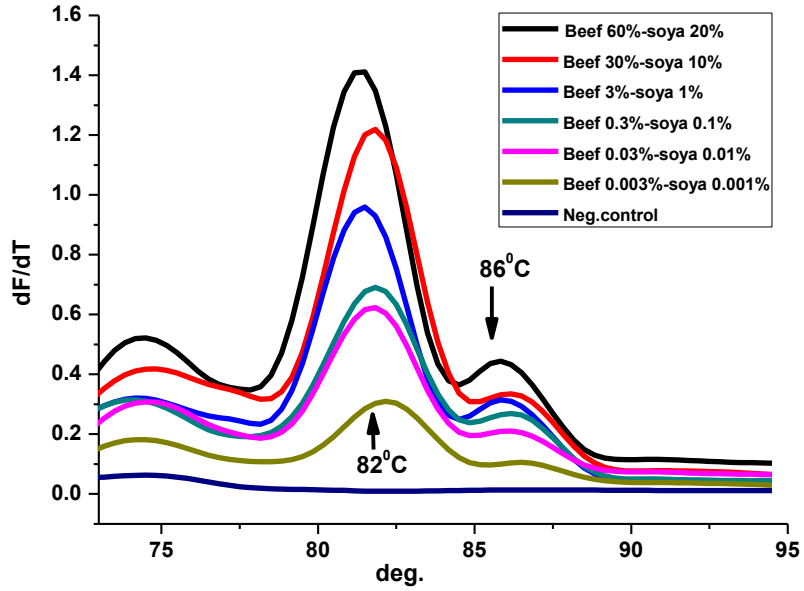


Figure 4.25 Determination of the relative sensitivity by using multiple DNA of beef, soybean and poultry in different ratios. Peaks at tm: 82°C correspond to amplicons obtained with 60%, 30%, 3%, 0.3%, 0.03 and 0.003% bovine DNA against a background of 90 ng soybean and poultry DNAs. Similarly, peaks at tm: 86°C correspond to amplicons obtained with 20%, 10%, 1%, 0.1%, 0.01%, and 0.001% soybean DNA against a background of 90 ng soybean and poultry DNAs. NC: negative control.

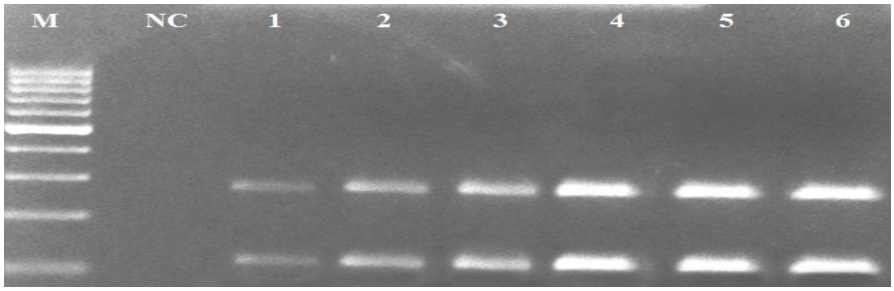


Figure 4.26 Agarose gel electrophoresis of EMRT-PCR products. M: 100 bp marker; NC: negative control; 1: 20% soybean-60%beef; 2: 10% soybean-30%beef; 3:1% soybean-3%beef; 4:0.1% soybean-0.3%beef; 5:0.01% soybean-0.03%beef; 6:0.001% soybean-0.003%beef.

4.9.4 Application of the EMRT-PCR assays to commercial sausages

The application of the EMRT-PCR assays to commercial sausages has been demonstrated in Figures 4.27-4.29 and Table 4.6. The results are shown in Figure 4.27 which indicated that 60% bovine samples were contaminated with soybean residuals, which were labeled as 100% beef and poultry mixed. These residuals are not in accordance with the ingredients labeled by the producer. With regard to commercial soybean and poultry mixed sausages, 60% species did not match with claimed species in label by the assay analysis (see Figure 4.28).However, the commercial sausages samples which contain beef and soybean and poultry mixture, claimed constituents have been confirmed by the EMRT-PCR analysis (see Figure 3.29). The 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 food products to verify the origins of raw material.

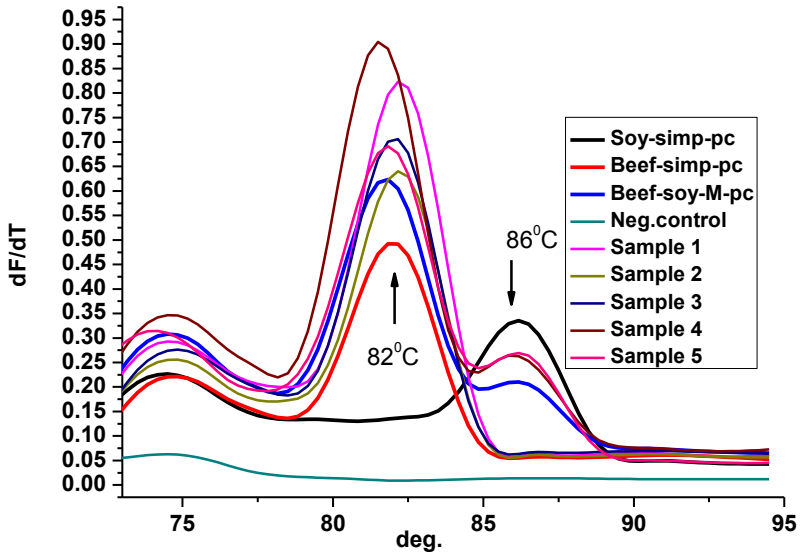


Figure 4.27 EMRT-PCR with multiple DNA from sausage mixed with beef and poultry using beef specific primer pairs. Peaks (tm: 82°C) correspond to amplicons obtained with beef DNA. Other peaks (tm: 86°C) correspond to amplicons obtained with soybean DNA which is contamination.Beef_simplex_positivecontrol, Soybean_simplex_positive control, Poultry/bovine_multiplex_positive control, NC: negative control.

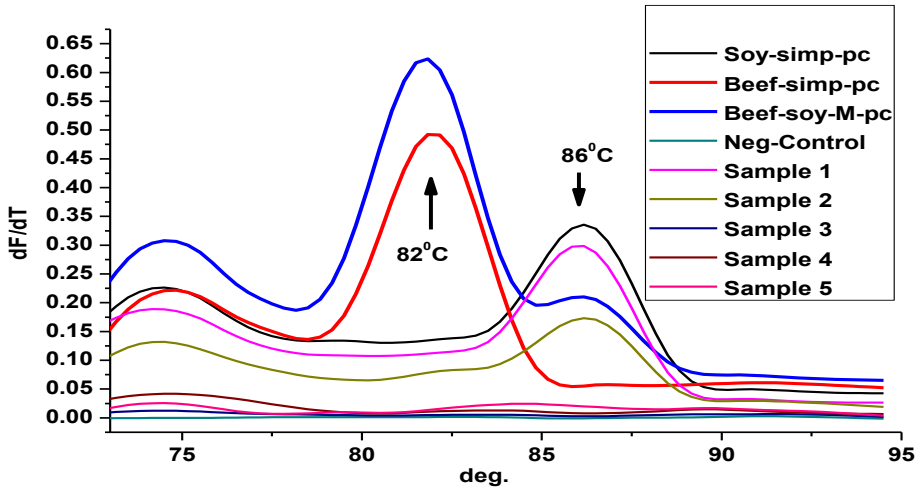


Figure 4.28 EMRT-PCR with multiple DNA from sausage mixed with soybean and poultry using soybean specific primer pairs. Peaks (tm: 86°C) correspond to amplicons obtained with beef DNA. Other peaks (tm: 82°C) correspond to amplicons obtained with beef DNA. Beef_simplex_positive control, Soybean_simplex_positive control, Poultry/bovine_multiplex_positive control, NC: negative control.

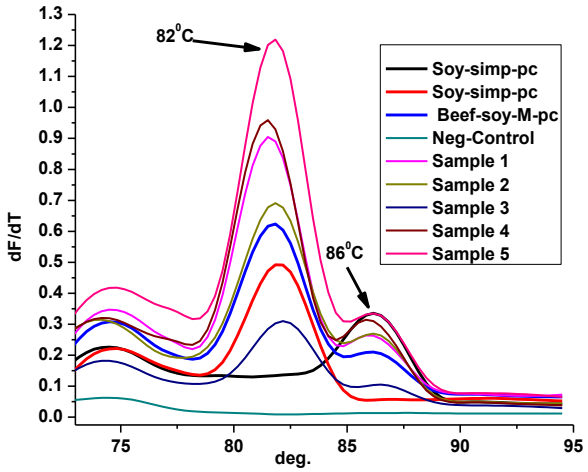


Figure 4.29 EMRT-PCR with multiple DNA from sausage mixed with beef, soybean and poultry using beef and soybean specific primer pairs. Peaks (tm: 82°C) correspond to amplicons obtained with beef DNA. Other peaks (tm: 86°C) correspond to amplicons obtained with soybean DNA. Beef_simplex_positive control,Soybean_simplex_positive control, Poultry/bovine_multiplex_positive control, NC: negative control.

Table 4.6 Results of EMRT-PCR performed on commercial sausages.

Products	Labeled	Results
Sausages , n® : 5	beef+ poultry	Beef, soybean
Sausages , n® : 5	poultry	soybean
Sausages , n® : 5	beef+soybean+poultry	beef+soybean

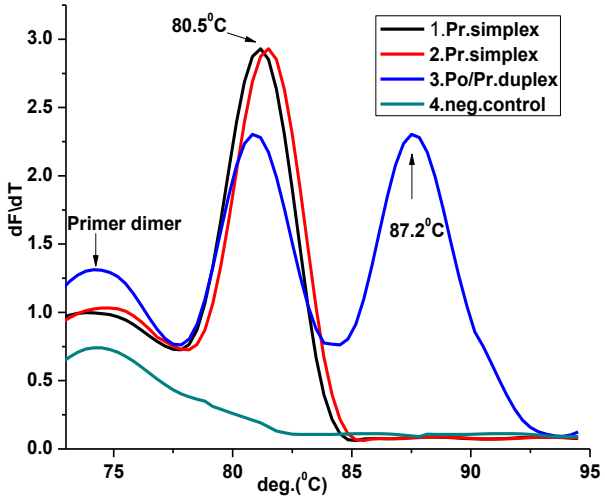
n® : number of samples

4.10 EVAGREEN MULTIPLEX REAL TIME PCR (EMRT)

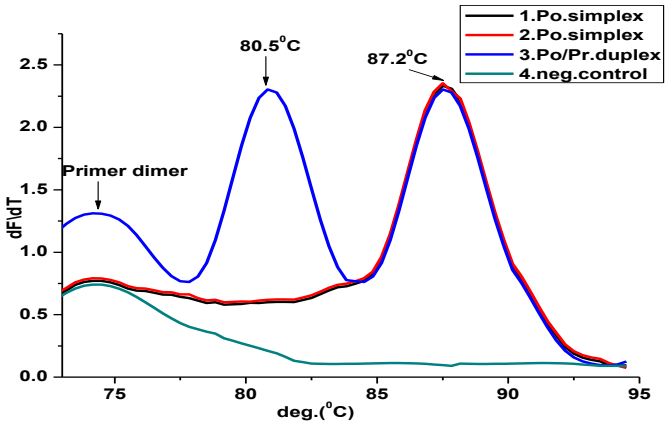
4.10.1 Development of EMRT-PCR

The primers for poultry and pork were mixed for the multiplex reaction. The templates were amplified in the real-time PCR followed by a melting curve analysis using EvaGreen and the Corbett Rotor Gene Analyzer System. The addition of amplicons in the same reaction was verified in a graph representing the changes in fluorescence as a function of time (dF/dT) versus the temperature of the reaction products. Pork and poultry amplicons were easily distinguished through specific tm values due to the different length and base compositions of two amplicons. The EMRT-PCR resulted in a single curve with two peaks as shown in Figures 3.30. These peaks formed at a specific location on the temperature axis at 80.5 °C for pork and 87.2 °C for poultry.

A



B



C

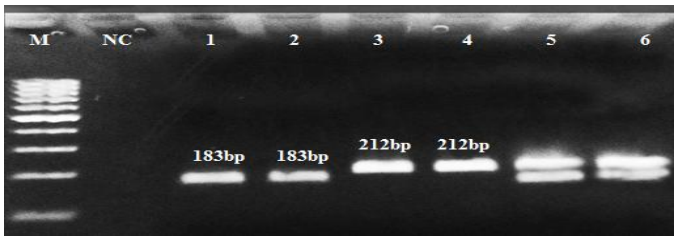


Figure 4.30 Specificity of the EMRT-PCR assay with fluorescence melting curve for pork and poultry in EvaGreen multiplex real-time PCR performed with Corbett Rotor Gene Analyzer. Melting temperature (tm) profiles of pork and poultry; (a) Pork_simplex positive control, tm: 80.5°C; Poultry/pork_multiplex positive control (multiplex amplification), tm: 80.5°C & 87.2°C; (b) Poultry_simplex positive control, tm: 87.2°C; Poultry/pork_multiplex positive control (multiplex amplification), tm: 80.5°C & 87.2°C; (c) agarose gel electrophoresis(cross check) of EMRT-PCR products; M; 100bp ladder, 1&2; poultry simplex positive control, 3&4; pork simplex positive control, 5&6; pork/poultry multiplex positive control, NC; Negative control.

4.10.2 Specificity of EMRT-PCR system

For the multiplex determination of pork and poultry tissues in pet foods, a primer specific to pork was designed Lahiff et al. (2001), and a poultry primer was designed by Dalmasso et al. (2004) were used. Both primers were used to develop amplification conditions. The specificity of the primers was tested for seven livestock species: pork, horse, soybean, bovine, sheep, chicken and turkey. It was verified that primers specific to the species of bovine and poultry showed no cross-reaction with any of the non-target species. Specificity of the multiplex assay was developed by carrying out melting curve analysis. This multiplex real-time PCR assay was specific for each species investigated with a little changeable melting temperature (tm). The tm values of pork and poultry genes were recorded as 80.5 ± 0.3 °C and 87.2 ± 0.4 °C. EMRT-PCR yields were run on 2% agarose gel stained with ethidium bromide to crosscheck. An agarose gel electrophoresis of the PCR products showed that pork and poultry samples produced clear bands of the expected size of 212 bp and 183 bp, respectively (see Figure 4.30).

4.10.3 Sensitivity of the EMRT-PCR system

To determine the sensitivity and linearity of the real-time PCR technique, the genomic DNA was obtained from each target species starting from the 50 ng/ul target DNA. Experiments with samples of decreasing concentration indicated that pork and poultry DNA concentrations as low as 0.0001 ng/ul still yielded an amplicons signal upon EMRT-PCR (Figure not shown). Amplification reactions with mixtures of pork and poultry DNA samples further demonstrated that as little as 0.003% pork and 0.001% poultry DNA could be detected against a background of 50 ng poultry and 50 ng pork DNA, respectively (see Figure 3.31).

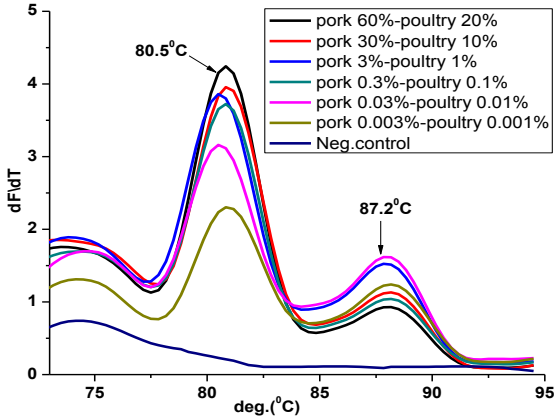


Figure 4.31 Determination of the relative sensitivity by using multiple DNA of pork, poultry and beef in different ratios. Peaks at tm: 80.5°C correspond to amplicons obtained with 60%, 30%, 3%, 0.3%, 0.03 and 0.003% pork DNA against a background of 100 ng pork, poultry and beef DNAs. Similarly, peaks at tm: 87.2°C correspond to amplicons obtained with 20%, 10%, 1%, 0.1%, 0.01%, and 0.001% poultry DNA against a background of 100 ng pork, poultry and beef DNAs. NC: negative control.

4.10.4 Application of the EMRT-PCR assays to commercial pet foods

The application of the EMRT-PCR assays to commercial pet foods has been demonstrated in Figures 4.32-4.34 and Table 4.7. The pet food samples which contain beef and poultry, claimed constituents have been confirmed by the EMRT-PCR analysis (see Figure 4.32). The results are shown in Figure 4.33 which indicated that 3/5 beef samples were contaminated with poultry residuals, which were labeled as 100% beef. These residuals are not in accordance with the ingredients labeled by the producer. Similarly, the results are shown in Figure 4.34 which indicated that 2/5 pork samples were contaminated with poultry residuals, which were labeled as 100% pork. The assays applied in this research have a high potential as a molecular tool that can be used in quality control laboratories for the verification of contaminated food products.

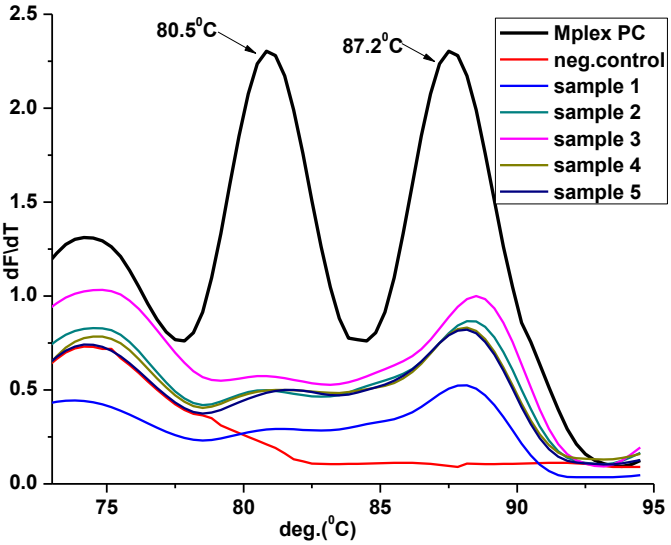


Figure 4.32 EMRT-PCR with multiple DNA from pet foods contained beef and poultry using pork and poultry specific primer pairs. Peaks (tm: 87.2°C) correspond to amplicons obtained with poultry DNA. Other peaks (tm: 80.5°C) correspond to amplicons obtained with pork DNA. Poultry/pork_multiplex positive control, NC: negative control.

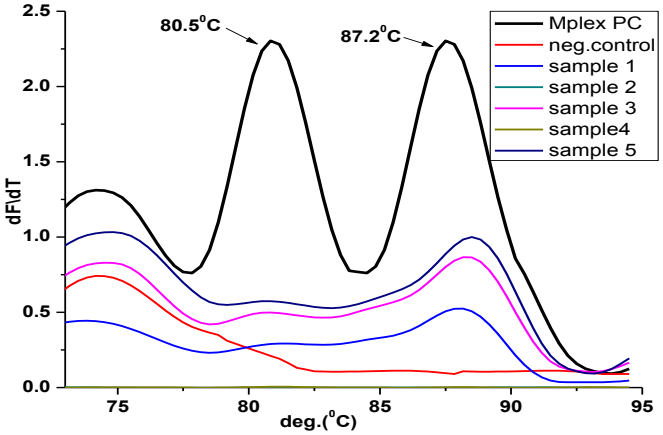


Figure 4.33 EMRT-PCR with multiple DNA from pet foods contained beef using pork and poultry specific primer pairs. Peaks (tm: 87.2°C) correspond to amplicons obtained with poultry DNA. Other peaks (tm: 80.2°C) correspond to amplicons obtained with pork DNA. Poultry/pork_multiplex positive control, NC: negative control.

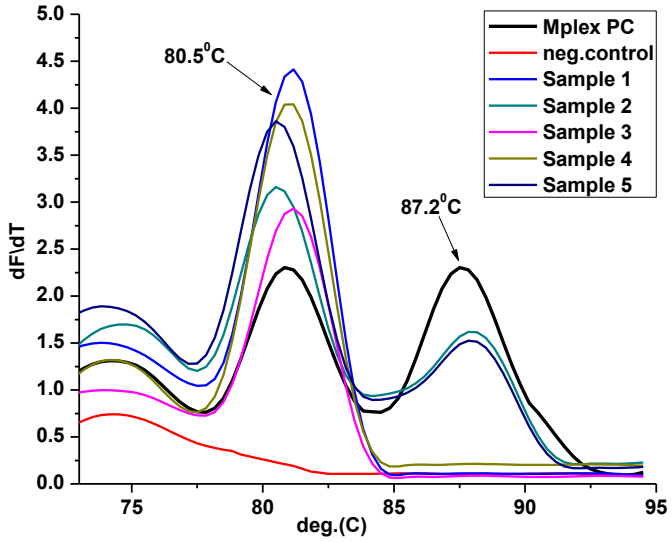


Figure 4.34 EMRT-PCR with multiple DNA from pork food samples using pork and poultry specific primer pairs. Peaks (tm: 80.5°C) correspond to amplicons obtained with pork DNA. Other peaks (tm: 87.5°C) correspond to amplicons obtained with poultry DNA which showed poultry contamination. Poultry/pork_multiplex positive control, NC: negative control.

Table 4.7 Results of EMRT-PCR performed on Commercial Pet Foods

Products	Labeled	Results
Pet Food , n® : 5	Poultry	Poultry
Pet Food , n® : 5	Beef	Poultry
Pet Food , n® : 5	Pork	Pork, poultry

n® : number of samples

CHAPTER 5

DISCUSSION

Transmissible Spongiform Encephalopathies (TSEs) are fatal neuro-degenerative diseases which referred as bovine spongiform encephalopathy (BSE) or ‘‘mad cow disease,’’ in bovine, variant of Creutzfeldt–Jakob disease in human. To minimize the risks of TSEs to humans and animals, the European Food Safety Authority adopted measurement to restrict the fish meal directly or indirectly in ruminant feed [14]. It is strongly suspected to have arisen from feeding cattle with rendered protein supplements derived from scrape-infected sheep and goat tissues and its spreading strictly correlated with the absence of stringent control on rendering process [15]. After putting ban on cattle feed enriched with ruminant derived protein, contaminated with infected material and intra-species recycling has decreased the BSE incidence in many countries. Similarly, the European Union (EU) has introduced restrictions in the production and use of meat and bone meal in farm animal’s feed [16-18]. Later on the Annexe IV in Regulation 2003/1234/EC amended the TSE Regulation, in the sense that all animal proteins from farmed animals are prohibited for the use in feedstuffs of farmed animals, due to the lack of animal-specific detection methods.

The authentication of foodstuff and feedstuffs is a great importance in terms of the consumers’ rights, unfair competition and medical requirements (food allergies). Some individuals have frequent adverse reactions (allergies) towards beef meat and soybean proteins. Species identification of food and feed products has also importance because of adulteration/substitution [48.82]

Food adulteration is defined as the act of intentionally corrupting the quality of food offered for sale either by the admixture or substitution of inferior substances or by the removal of some valuable ingredient. Adulterations of food and feed products are

the replacement of expensive food and feed products by non-declared low quality products. Commonly, less expensive poultry, fish, soybean, pork and horse species are mixed with other species such as goat, sheep and cow to make food and feed products that is of less quality and non-declared in label but sale as pure one [35,50].

The other important point is that adulteration causes unfair competition between food and feed producers. For example, cheaper, non-declared poultry, soybean and fish products are mixed with expensive one and leads to decrease the raw material cost so that the company sells the product cheaper than the other company. That is why several quantitative and qualitative analytical methods are used to identify meat species used in feed and food products.

The available methods of species identification from foodstuff and feedstuffs based on various forms of electrophoresis and use of immune sera in agar gel diffusion [83]. Some of such methods of animal tissue identification are agar gel diffusion, passive haemagglutination, immuno-electrophoresis, counter immunoelectrophoresis and enzyme-linked immunosorbent assay [84]. Although, electrophoretical and chromatographic methods are very popular, but they have many disadvantages such as difficult optimization procedures, time consuming, high cost, low specificity and sensitivity. In recent past, DNA as a source of information has been used for speciation of animal species because DNA have significant amounts of sequence variation in closely related species, more thermostable than many proteins and easier to retrieve from low-quantity and/or degraded DNA from samples [85]. Some of the molecular approaches applied for meat species identification include RAPD–PCR (Calvo et al., 2001), RFLP analysis [19], Simplex PCR [86]. Real-time PCR is probably the most used quantitative DNA-based method these days. However, the high cost of the equipment and reagents is still a drawback for the application of this technique in most laboratories [87, 88]. Alternatively, multiplex PCR is a rapid, economical and simple approach to use DNA for commercial analysis and surveillance of foods [50,30] Alessandra Dalmasso et al. developed Real-Time PCR assay for simultaneously detecting cow and buffalo milk in mozzarella cheese [50].

The disadvantage of most real-time PCR applications is that the high cost derived of specific fluorescent probes [89]. To decrease this limitation, SYBR Green and

EvaGreen were introduced for the detection of PCR products without the need for probes linked to fluorescent molecules [89, 87].

Some scientists anticipated that MRT-PCR with SYBR Green/EvaGreen fluorescence dye cannot be possible. Although multiplexing reactions cannot be performed with SYBR Green, specificity can be achieved by careful primer design and reaction optimization, which can be confirmed from melt curve analysis [41, 81, 19]. TaqMan Real-time PCR was used by many scientists in MRT-PCR. The similarity of each species-specific amplicons in relation to fluorophor-specific TaqMan probes would make these assays convenient to multicolor multiplex detection, whereas DNA intercalating dye detection would not possible [1].

Although there are few reports with microorganisms described the use of a multiplex fluorogenic PCR [81]. Şakalar and Abasıyanık applied and optimized a SDRT-PCR (SYBR Green Duplex Real-time-Polymerase Chain Reaction) technique for detection of ruminant and poultry materials in some industrial meat products (Şakalar and Abasıyanık, 2011) which was developed to improve an assay that can combine the two advantages of real-time PCR and multiplex PCR together for animal gene detection and identification more quickly but they used larger amplicons sizes.

In this thesis, we optimized modern PCR Techniques such as qualitative simplex and multiplex PCR and also developed a novel MRT-PCRs using SYBR Green and EvaGreen dyes in order to detect food and feed origins in foodstuff and feedstuffs. Using the intercalating fluorescence dye, SYBR Green I/EvaGreen and the Corbett Rotor Gene Analyzer system, the accumulation of amplicons in the reaction can be monitored over time. SYBR Green I and EvaGreen dispense with the need for analyzing PCR products on time consuming agarose gels. After PCR amplification, the Corbett Rotor Gene Analyzer continuously monitors the decrease of fluorescence resulting from the release of EvaGreen/SYBR Green I during DNA melting point analysis by slowly increasing the temperature. The Tm of specific amplicons and unique shape of the melting peak can be used to differentiate the target genes and identify them.

Numerous factors have influenced the successful PCR amplification e.g. quality and quantity of DNA, specificity of primers, amplicons sizes, etc. The quantity and

quality of DNA can be affected due to high temperature, microorganism and chemical treatments [88]. In DNA extraction step of our study, the amount of target DNA in autoclaved meat, food and feed products was lower than the amount of fresh meat DNA. This could indicate that the DNA was of low quality, either due to degradation caused by food processing or the presence of PCR inhibitor which were not sufficiently removed in the purification step.

As we mentioned above, food and feed processing especially heat-treatment causes DNA degradation. To validate the commercial feed samples such as ruminants feed, raw meat of each species (cow, goat, sheep, poultry, horse, pork and fish) were autoclaved according to the European legislation (133°C at 300 kPa for 20 min) and we focused smaller target sequences (85, 93, 119, 100, 142, 183, 212 and 224 bp for horse, cow, sheep, soybean, goat, poultry, pork and fish respectively) for avoiding any problem in DNA amplification step.

The specificity of the primers is also an important component in accurate identification of species. Species specific primers targeting region of mitochondrial genome [82,90] 12S rRNA [91]have been developed for detection of adulteration of food and feed products by detecting source of DNA fragments in a simplex or multiplex PCR. In the present study, mitochondrial DNA was targeted for species specific identification of food and feed products. This mitochondrial DNA was selected because of its variability among species and high copy number of mitochondrial DNA as compared to nuclear DNA. Three primers including a cow specific, a sheep specific and a goat specific were used for amplifying cow and sheep or goat specific segment from mitochondrial DNA region. The specificity of primers was confirmed with preliminary PCR experiments where the species specific primers did not show any amplification in other closely related species.

The result of our study suggests that primers given can be used for species specific identification of food and feed products in simplex PCR. However, the primer combinations selected for this study is well applicable for specific identification of food and feed products in multiplex PCR assay as well.

In this thesis, the Modern PCR methods (Tetraplex PCR, Hexaplex PCR and Heptaplex PCR) depict the development and application of multiplex PCR to detect

bovine, poultry, fish, sheep, goat, horse, soybean and pork materials in foodstuff and feedstuffs in a single reaction step that highly decreases the cost of tests. The detection limit of these assays (Tetraplex PCR/ Heptaplex PCR for feedstuffs, Tetraplex PCR/ Hexaplex PCR for feedstuffs and foodstuffs) on reference samples was 0.01% which indicating a high sensitivity of this method. The detection limits using previously reported methods showed 1% for ruminants DNA [92, 93]. Whereas, [94 and 95] described PCR assays for the detection of bovine-derived material in animal feedstuffs with the detection limit of 0.125%. In other words, the low range detection limit of our PCR assays we report here exceeds the previously reported assays by a minimum level of 0.01% (see Figures. 4.4, 4.8, 4.11, 4.14).

Disadvantageously, obviously, Multiplex Qualitative PCR is not as sensitive as real time PCR. Because of this drawback, we tried to develop a real-time PCR technique by SYBR Green and EvaGreen to identify the presence of animal material in foodstuff and feedstuff samples.

In this study, we optimized SDRT-PCR and EMRT-PCR assays were applied to commercial sausages, pet foods and ruminants feed and it showed the clear evidence about presence of those species which are not indicated on the label. The amplification of DNA of standard food and feed samples allowed the detection limit of 0.001% of sausages, pet food, ruminants feed (sensitivity of PCR assay) by means of a Real-time PCR (see Figure 4.17). These results showed high detection limit than previously reported as Jerilyn A. Walker et al, also used it to make a quantitative analysis according to the gel electrophoresis results of amplicons. They found out that bovine DNA was detected at 0.005 %(0.5 pg), porcine DNA was detected at 0.0005% (0.05 pg), and chicken DNA was detected at 0.05% (5 pg) in a 10-ng mixture of bovine, porcine, and chicken DNA templates by this method. We also optimized a similar method and could detect 0,001% of amplicons by bovine, poultry, soybean, pork (see Figures 4.19, 4.26, 4.31). We also tested all samples with bovine, poultry, soybean and pork primers, and saw that none of the samples except pork samples declared on the labels was contaminated by poultry (meats) and soybean proteins.

In this study, we adapted duplex SYBR Green I real-time PCR assay and EvaGreen Multiplex real time PCR which appears to be an encouraging tool for rapid, sensitive, specific and accurate identification of animal genes in foodstuff and

feedstuffs. The use of a simple and less expensive EvaGreen and SYBR Green I format with Tm analysis of PCR products is easier and faster to perform compared with qualitative PCR diagnostic approaches. Determination of melting points was very reproducible in our experiments.

In conclusion, the Modern PCR methods (Tetraplex-PCR, Hexaplex-PCR, Heptaplex-PCR, SDRT-PCR and EMRT-PCR) proposed in this study represent rapid and efficient alternative methods applicable on a routine basis for the control of feedstuff and foodstuffs in which cow, goat, horse, pork, soybean, fish and sheep species need to be detected. The use of our assay for the identification of plant and animal DNAs in food and feed products will provide additional molecular approach for outbreak investigation.

CHAPTER 6

CONCLUSIONS

We developed and optimized modern PCR techniques (MRT-PCRs and MQ-PCRs) for rapid multiplex analysis of food and feed products. They are promising techniques for inexpensive, rapid, sensitive, specific and accurate identification of animal DNAs in the processed food and feed products. These techniques are potentially reliable techniques for detection of TSEs origin, strict adulteration, labeling regulation and Halal authentication in foodstuff and feedstuff. MRT-PCRs are almost seven folds cheaper than probe-based methods. In addition MRT-PCRs are methods that clarified the origins of positive result whether it is due to adulteration of the product or inadequate handling during manufacture and useful tools in the food and feed industries.

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