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A highly sensitive and specific tetraplex PCR assay for soybean, poultry, horse and pork species identification in sausages: Development and validation

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ABSTRACT

A tetraplex PCR assay was developed for a rapid and reliable identification of horse, soybean, poultry, and pork species in sausages simultaneously. The method merges the use of horse (*Equus caballus*), soybean (*Glycine max*), poultry (*Gallus gallus*), and pork (*Sus scrofa*) specific primers that amplify small fragments (horse; 85 bp, soybean; 100 bp, poultry; 183 bp and pork; 212 bp) of the mitochondrial cyt b, lectin, 12S rRNA and ATPase subunit 6 genes respectively. Good quality DNA was isolated from reference sausage to optimize the assay. Tetraplex analysis of the reference sausage samples showed that the detection limit of the assay was 0.01% for each species. Taken together, all data indicated that this tetraplex PCR assay was a simple, rapid, sensitive, specific, and cost-effective detection method for horse, soybean, poultry, and pork species in commercial sausages.

1. Introduction

Food composition and authenticity assessment is an important concern to protect the consumers from illegal or unwanted substitution; for economic, religious and health concerning reasons. Chicken, pork and horse meat are being used as a substitute ingredient for red meat, whereas, vegetable proteins (soybean) are used as an alternative of muscle proteins, due to their low cost of production (Arslan, Irfan-Ilhak, & Calicioglu, 2006; Mane, Mendiratta, & Tiwari, 2009). Soy allergy is an arising public health concern among food allergies as even minute quantity of soy, may trigger allergic reactions in children and adults (Abdullah, Radu, Hassan, & Khair Hashim, 2006; Poms, Anklam, & Kuhn, 2004). Muslim and Jewish populations avoid consumption of pork and horse meat, even in minute quantities, due to their religious faiths (Teletchea, Maudet, & Hanni, 2005). In this background, there is a need of a rapid, economic and highly sensitive method for identification of meat species in sausages.

Many analytical methods; chemical, electrophoretical, chromatographic, and immunological have been used for soy, poultry, pork and horse species identification in foods, but each method having its own limitations (Arslan et al., 2006; Ballin, Vogensen, & Karlsson, 2012; Espineira, Herrero, Vieites, & Santaclara, 2010). In parallel, several researchers have employed conventional gel electrophoresis-based PCR-detection for qualitative analysis of soy protein and meat species in sausages (Che Man, Aida, Raha, & Son, 2007; Miguel & Enrique, 2014; Murugaiah et al., 2009; Nakyinsige, Che Man, & Sazili, 2012). In contrast to conventional PCR techniques, real-time PCR-approaches identify even minute traces of soy protein and meat species in sausages (Espineira et al., 2010; Miguel, García, González, Hernández, & Martín, 2005; Safdar & Abasıyanık, 2013a, 2013b). However, the high cost of the equipment and reagents is a matter of concern for applying this technique in most laboratories (Safdar & Abasıyanık, 2013a, 2013b; Zhang, Fowler, Scott, Lawson, & Slater, 2007). Alternatively, multiplex PCR is a rapid, economical and simple approach for commercial analysis of sausages (Safdar & Abasıyanık, 2013a, 2013b; Sónia, Joana, Isabel, Beatriz, & Oliveira, 2010).

Therefore it is the urge of time for the requirement of a technique which endorses simple, cost-effective and prompt methods to use DNA-based commercial analysis and surveillance of sausages. Some researchers have applied simultaneous PCR for the detection of meat species in sausages (Di Pinto, Forte, Conversano, & Tantillo, 2005; Sónia et al., 2010). But as far as our knowledge, there has been no study related to tetraplex PCR for sensitive and specific horse, soybean, poultry and pork species identification in sausages simultaneously. That's why the tetraplex PCR assay has been reported to specifically identify horse, soybean, poultry and pork by using small fragments of

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DNA in simultaneous reaction (one tube) in sausages. The objective of the present study was to develop a tetraplex PCR assay which shows a potential tool for rapid, specific, sensitive and cost-effective detection of small fragment of horse, soybean, poultry and pork mitochondrial DNA origins in sausages.

2. Material and methods

2.1. Preparation and selection of samples

To validate the tetraplex PCR, the reference sausage samples were prepared from beef, chicken, horse, pork meats and soybean protein, and additives, such as spices and beef meat in our laboratory (Table 1). To determine the detection limit of soybean, poultry, horse and pork in reference sausages, samples were mixed in the range of 20% to 0.01%. Twenty commercial sausages were obtained from local supermarkets in Turkey. They all were directly transported to the Genetic Research Laboratory of Fatih University and stored at −20 °C until the extraction of the DNA in order to prevent the enzymatic degradation of DNA.

2.2. DNA isolation

Total DNA was extracted from samples following manufacturer's instructions using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured with a NanoDrop2000 spectrophotometer (NanoDrop2000, UV–Vis spectrophotometer, USA). The samples were exposed to ultraviolet light at 260 and 280 nm. 260:280 was used to calculate the quantification of nucleic acids by the following formula: DNA concentration = OD260 × extinction coefficient (50 μg/ml) × dilution factor.

2.3. Primers

Horse, soybean, pork and poultry primers published respectively by Koppel, Ruf, and Rentsch (2011), Zhang et al. (2007), Lahiff et al. (2001) and Dalmasso et al. (2004) were used (Table 2). All primers used in this study were synthesized by the Metabion Company (Germany). To check the specificity of each primer BLAST program was used.

2.4. Simplex and tetraplex PCR

For the both simplex and tetraplex detection of species, PCR amplification was performed in a final volume of 25 μl (5× HOT FIREPol EvaGreen® qPCR Mix Plus (ROX), Solis Bio Dyne, 10 pmol of pork, poultry, soybean and horse primers of each species and 120 ng of DNA template). Amplification was performed in a Thermo cycler Techne with the following cycling conditions; after an initial heat denaturation step at 94 °C for 10 min, 35 cycles were programmed as follows: 94 °C for 30 s, 59.7 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 5 min. PCR amplified products were analyzed by electrophoresis on 2% agarose (Helicon, USA) gel run in 0.5× TBE buffer (Trisbase, boric acid, NaEDTA) for 50 min at 100 V and stained with ethidium bromide (10 ng/ml) for 20 min. The agarose gel was visualized under UV light (Vilber Lourmat BP 66, France) and image was taken by a camera (Sony Cyber shot DSC T520).

Table 1
Composition of reference sausages for sensitivity.

Horse (%)	Soybean (%)	Poultry (%)	Pork (%)
20	20	20	20
10	10	10	10
1	1	1	1
0.1	0.1	0.1	0.1
0.01	0.01	0.01	0.01

3. Results and discussion

3.1. DNA extraction

The results showed that extracted DNA was suitable for PCR amplification. The DNA extraction method was considered satisfactory and was able to remove PCR inhibitors, which could interfere with PCR reaction. For example, polysaccharides and polyphenols are common PCR inhibitors in plant sources such as proteins (Di Pinto et al., 2007) while, oil and fats are common components of many food ingredients which are known to inhibit polymerases (Arlorio, Cereti, Coïsson, Travaglia, & Martelli, 2007). The purity and yields of the total DNA extract obtained from reference and commercial sausage samples were high (purity = A260/A280 and 260/230 ratio ranged between 1.8 and 2.0 and yield = 40–50 ng/μl).

3.2. Specificity of simplex and tetraplex PCR

In an elementary phase of this research, simplex and tetraplex PCRs were done by using the DNA extracted from reference sausages. The PCR product was run on agarose gel for visualizing the proper amplification. The amplification of total DNA 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 (Fig. 1).

3.3. Sensitivity

In order to find out the sensitivity of our employed method, tetraplex PCR assays were carried out for soybean, poultry, horse, and pork species identification in reference sausages. Reference sausages were prepared by homogenized mixing in appropriate ratios of 0.01 to 20% (Table 1) of each species (horse, soybean, poultry and pork) meats in beef meats to test the sensitivity of the assay for commercial sausages. The PCR product was run on agarose gel in order to check the sensitivity. Reference sausage sample sensitivity results showed that the sensitivity threshold was 0.01% for each species (Fig. 2).

3.4. Primer specificity

The primer specificity results revealed that no cross-reactivity was seen with respective species DNA (horse, poultry, pork and soybean) from other species DNA (sheep, goat, cow, donkey, fish, cat, dog, pig, buffalo, deer, mouse, rabbit, rat, wheat, maize and human). PCR products were not obtained for the samples of negative controls with any of the species-specific primer sets. Every test was repeated four times which gave reproducible results (data not shown).

3.5. Applying tetraplex PCR system on sausages

The application of the assays on commercial sausages has been depicted in Fig. 3A–D and Table 3, which show the accurate species composition of the submitted sausage samples. 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 sausage tested samples showed that 3/5 samples were contaminated with poultry origins while 2/5 samples were contaminated with soybean protein origin contents. Similarly, 3/5 samples of beef and poultry mixed sausages were also adulterated with soybean protein origin contents. Finally, the results of beef, poultry and soybean mixed sausage tested samples showed that 100% samples were verified correctly as labeled using tetraplex PCR assay.

These results revealed some interesting and shocking findings. The samples which were claiming to have 100% beef content were found