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Application of Quadruple Multiplex PCR Detection for Beef, Duck, Mutton and Pork in Mixed Meat

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Abstract In this study, we have developed quadruple multiplex PCR assay for meat (beef, duck, mutton and pork) in processed meat products. By mixing four primers in appropriate ratios could be identified by the PCR. A forward primer was designed on a conserved DNA sequence in the mitochondrial ND2 and 16S rDNA gene in sheep and duck genes, and reverse primers on species-specific DNA sequences for each species. PCR primers were designed to give different length fragments from the four meats. The products showed species-specific DNA fragments of 116, 212, 177 and 322 bp from beef, pork, mutton and duck. Optimal PCR conditions were established. The assay sensitivity under these conditions was 0.1ng, and its specificity was 100 %. The results of the study suggest that PCR represents a simple, efficient test method as a practical alternative for the rapid detection and identification of meat.

Keywords: *multiplex PCR; beef; duck; mutton; pork; identification*

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1. Introduction

The identification of species in food is becoming a very important issue concerning the assessment of food composition, which is necessary to provide consumers accurate information about the products they purchase. Consumers demand higher protection from falsely labeled meat products for a variety of economic, religious and health reasons, which are enhanced by the recent crisis in the meat sector [1].

Through survey [2,3,4] found that some of duck and pig meat adulterated in mutton and beef could be attributed either to a fraudulent addition or to a simple accidental contamination. This can have severe economic, ethical and medical repercussions [5]. To detect meat species in mixed samples, a variety of analytical methods have been used, mainly including high-performance liquid chromatography, electrophoretic techniques, and enzyme-linked immunosorbent assays [6], the used of SDS-PAGE to differentiate beef, horse and pork meat in cooked sausages [7], Polymerase chain reaction (PCR) based assays for species identification [8,9,10], and analysis of species-specific mitochondrial DNA sequences used the PCR to identification of meat species in food [11,12]. To date, a number of PCR-based methods for meat identification, including multiplex PCR [13], PCR-RFLP [14,15,16,17], Dot-ELISA [18], Taqman or SYBR GREEN Real-time PCR [19-26], a loop-mediated isothermal amplification (LAMP) [27], without prior DNA extraction used to direct PCR-DNA rapid and economical simultaneous identification of meat species [28] and used of multiplex PCR assay for the identification of animal species in feedstuffs [29,30,31,32].

The amount of "banned" inferior products would be low in commercially available meat products, therefore, our study was to develop a rapid, sensitive, and a reliable multiplex PCR method for simultaneously and accurately distinguish duck, pig, bovine and sheep meats in processed meat products.

2. Materials and Methods

2.1. Sample Preparation

Food samples from the Lanzhou (China) market were divided into three categories: raw meat samples of pork, beef (including meat from cattle and yak), duck and mutton (including meat from goat and sheep); cooked meats (To simulate steam cooking, samples of 5 g of whole raw meat were autoclaved 103.4 kPa pressure, 121°C temperature for 30 min); further processed foods, including sauced beef, canned beef, lamb shashlik, rolled meat, cooked bacon, dried meat floss, fried meat flakes and ham sausage, etc. DNA was extracted from these samples.

2.2. Primer Design

Mitochondrial sequences of bovine, pig, duck, ovine and caprine were downloaded from the GenBank database and aligned using MEGA 5.1 program (Lasergene software; DNAStar, Inc., Madison, Wisconsin, USA). The

GenBank accession numbers were as follows: duck (Anas platyrhynchos breed Beijing duck and Anas poecilorhyncha) EU755252.1, KF156760.1; ovine and caprine (Ovis aries and Capra hircus) NC_001941, NC_005044.2.

Specific primer sets with similar annealing temperatures were designed with Primer 6.0 on the basis of ND2 [the Ovis aries (sheep) NADH dehydrogenase subunit 2] and 16S rRNA (duck) gene sequences.The specific primers were synthesized by GenScript and verified bioinformatically by an Oligo 6.0 programmer. The sizes of the expected products and corresponding target genes are provided in Table 1.

Table 1. Species-specific primers sets

Primer	Target	Sequence (5'–3')	Expected product size (bp)	Reference
mcb398	cyt b	TACCATGAGGACAAATATCATTCTG	472	(Verma,et al [33])
mcb869		CCTCCTAGTTTGTTAGGGATTGATCG		
Mutton-F	ND2	CTCAACCACAACCACCCTAT	177	This study
Mutton-R		AGGGTGGGTAAGATAATGCTG		
Duck-F	16S	GCCACAAACAACAATAGTAAGC	322	This study
Duck-R		CCCGAGGTTCAGGTCTACTA		
Pork-F	cyt b	GCCTAAATCTCCCCTCAATGCTA	212	(Hongwei Gao,et al 2007)
Pork-R		ATGAAAGAGGCAAATAGATTTTCG		
Beef-F	cyt b	CGGAGTAATCCTTCTGCTCACAGT	116	[34]
Beef-R		GGATTGCTGATAAGAGGTTGGTG		

2.3. DNA Extraction

DNA was extracted from raw meat, meat products and cooked meats (raw meat were autoclaved 103.4 kPa pressure, 121°C temperature for 30 min) using CTAB (Hexadecyltrimethy Ammonium Bromide). Approximately 25 mg of ground meat was used for DNA extraction. Briefly, 25 mg of sample were digested in the CTAB lysing buffer, and the lysate was transferred into a 1.5 mL centrifuge tube. After centrifuging at 12,000 g for 5 min, Further DNA extraction procedures followed standard phenol-chloroform extraction method [35]. The extracted DNA was dissolved into 100µL of Tris-EDTA (pH 8.0) buffer. The genomic DNA was checked by using spectrophotometer taking O.D.260-280.

2.4. Meat Identification using PCR

A pair of universal primer (mcb398/mcb869) was used to amplify the cyt b gene. Primers sequences were as follows. PCR reaction 25 µL of 2×Taq Master Mix (Vazyme), 15 pmol of each primer and 100-150 ng template DNA. PCR was carried out in a Mastercycler® (Eppendorf, Germany). The cycling conditions included a single initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45 s (denaturation), 55°C for 45 s (annealing), 72°C for 1min (primer extension) and a final extension step at 72°C for 7 min. Negative controls (water) were included in each amplification, in order to verify the PCR efficiency and to detect contamination.

The multiplex PCR amplification can be influenced by many factors, including annealing temperature, primer concentration. Gradient PCR was used to establish the optimum reaction conditions for each of the parameters as listed: temperature, 55, 55.9, 57, 58.1, 59.4 and 60°C; primer concentration, 10, 15 and 20 pmol.

Amplification was conducted in 25 µL of 2×Taq Master Mix (Vazyme), primer mix (10, 15 and 20 pmol each) and appropriate template DNA. Multiplex PCR amplification was performed by adding mixture of new primers designed in the different ratios, initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 45 s (denaturation), 55-60°C for 45 s (annealing), 72°C for 1min (primer extension) and a final extension step at 72°C for 7 min. and performed an optimized annealing temperature and multiplex PCR step. Following amplification, 10 µL PCR solution was electrophoresed on 2.5% agarose gel (BBI) with goldview (0.5µg mL^{-1}) for 30 min at 120 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and visualized in a gel imaging system (Peiqing Scientific, Shanghai, China).

2.5. Multiplex PCR Specificity and Sensitivity of the Assay

The specificity of each species-specific primer was confirmed by amplification of 100 ng of donkey meat, fish, shrimp, mouse meat and chicken genomic DNA, and a negative control without DNA.

The sensitivity of PCR was assessed by a second method; firstly, the detection limit of the specific primers, and 1:10 serial dilutions were prepared. For each target meat, a base adulteration meat mixture was prepared by mixing meats of pork, beef, duck and mutton in equal quantity (25 mg each) DNA extraction. Ten fold serial dilutions of the template DNA were prepared starting with 100, 10, 1, 0.1, 0.01 and 0.001 ng per reaction and multiplex PCR was performed.

3. Results

3.1. Evaluation of DNA

Electrophoresis of DNA extracted from raw meat showed a high content of fragments **>** 2000 bp, whereas DNA extracted from autoclave-treated meat showed a majority of smaller fragments (≤750 bp) (A260/A280 ratio of 1.0–1.68) .Result DNA extracted from raw meat showed a high content of fragment>2000 bp, we can see from lines 2, 6, 12, 14. Whereas DNA extracted from autoclave-treated meat and product meat showed a majority of smaller fragments <750bp, lines1, 3, 4, 5, 7, 8, 9, 10, 11, 13, 15. (Figure 1). DNA sequence was reduced

to 1 100 bp at 100°C and 600 bp at 120°C [36,37]. The differentiation of animal species has proved to be difficult, particularly in samples of complex composition when subjected to heat, pickled and grilled [38]**.** On account of these were including large quantities of salts and preservatives. In this environment of living cells, which genome was substantial degradation. Ham sausage, canned beef and pork did not extract DNA [39].

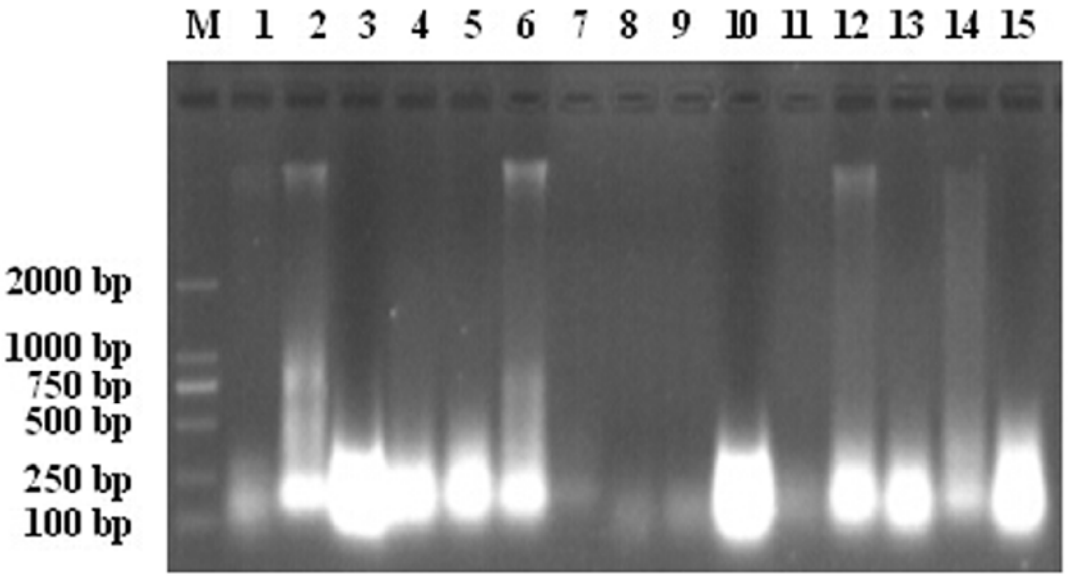

Figure 1. Gel electrophoresis of total genomic DNA extracted from: M, DL 2000 DNA Marker (Takara).1, smoked and cooked sausage (pork and beef); 2, raw pork; 3, heat treated (121°C 30 min) duck tissues; 4, heat treated (121°C 30 min) bovine tissues; 5, heat treated (121°C 30 min) pork; 6, raw yak meat; 7, ham sausage (beef); 8, canned beef; 9, canned pork; 10, diced chicken; 11, spicy chilli chicken; 12, raw mutton; 13, heat treated (121°C 30 min) sheep tissues; 14, raw beef; 15, heat treated (121°C 30 min) yak tissues

3.2. PCR Amplification and Optimization

Universal primers (mcb398/mcb869) used in this study amplified a 472 bp fragment of mitochondrial gene in all fifteen meat samples (Figure 2). Result universal primers (mcb398/mcb869) did not amplify from ham sausage (beef), canned beef and canned pork. On account of these were including large quantities of salts and preservatives. In this environment of living cells, which genome was substantial degradation [40,41].

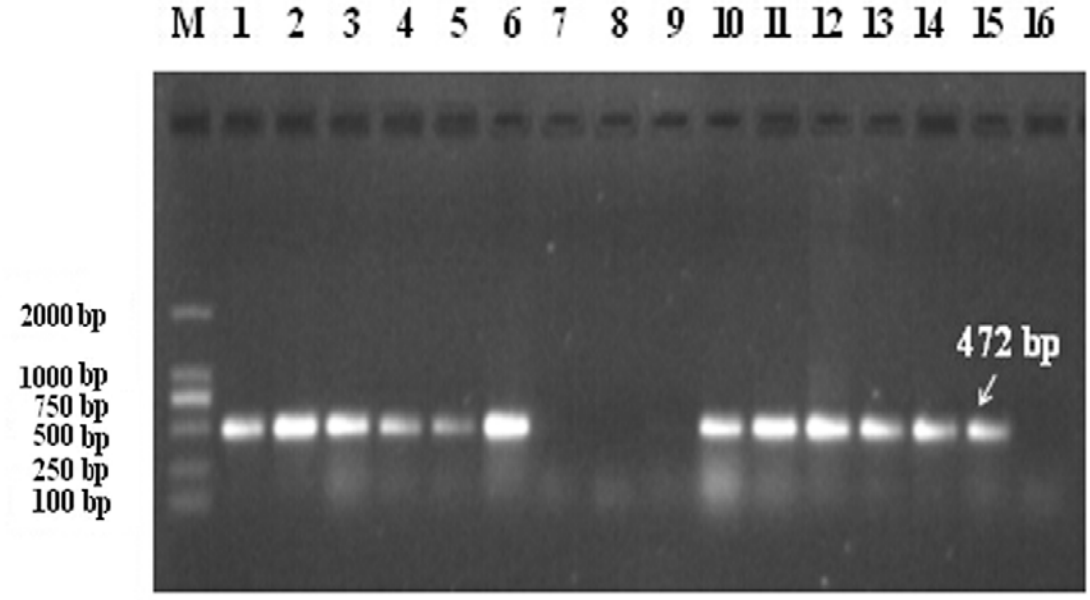

Figure 2. Universal primers (mcb398/mcb869) amplified a 472 bp fragment of mitochondrial gene: M, DL 2000 DNA Marker (Takara). 1, smoked and cooked sausage (pork and beef); 2, raw pork; 3, heat treated (121°C 30 min) duck tissues; 4, heat treated (121°C 30 min) bovine tissues; 5, heat treated (121°C 30 min) pork; 6, raw yak meat; 7, ham sausage (beef); 8,canned beef; 9, canned pork; 10, diced chicken; 11, spicy chilli chicken; 12, raw mutton; 13, heat treated (121°C 30 min) sheep tissues; 14, raw beef ; 15, heat treated (121°C 30 min) yak tissues; 16, control reagent

The optimized Multiplex PCR analyses were carried out in a final volume of 50 µL, including 25 µL of 2×Taq Master Mix, 4µL quadruple primer mixtures (respective,15 pmol), 100 ng of DNA template and 19 µL distilled water. Amplification was carried out in a Mastercycler® (Eppendorf, Germany) with denaturation

at 94 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min, with a final extension step at 72 °C for 7 min, and held at 4 °C. The PCR products were electrophoresed on 3 % (w/v) agarose gel. The application of primers pork, beef, duck and mutton to confirm reactivity among the multiplex PCR, the specific samples from cooked meat obtained 212 bp for pork, 116 bp for beef, 322 bp for duck and 177bp for mutton (Figure 3). Result the application of primers pork, beef, duck and mutton to confirm reactivity among the multiplex PCR, the specific samples from cooked meat obtained 212 bp for pork, 116 bp for beef, 322 bp for duck and 177bp for mutton, the primers successfully generated specific fragments.

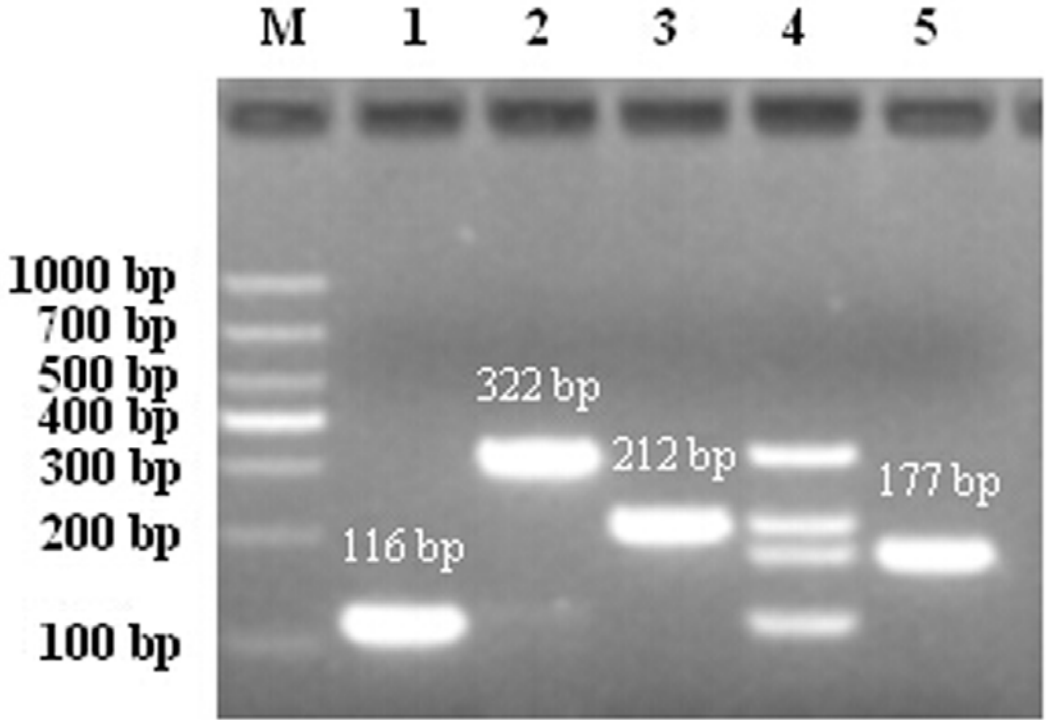

Figure 3. The multiplex PCR: M, DL 1000 DNA Marker (Takara). 1, beef; 2, duck; 3, pork; 4, combined duck, pork, mutton and beef; 5, mutton

3.3. Species Identification by the Multiplex PCR

To rule out the possibility of obtaining false positives, multiplex PCR was performed to improve the method. As shown in Figure 4, this approach was specific in detection of various combinations of target meat species without cross reaction with donkey meat, fish, shrimp, mouse meat and chicken. Result the Multiplex PCR was used to improve amplification conditions. Specificity primers were tested for five meats: donkey meat, fish, shrimp, mouse meat and chicken. It was tested that primers specific to the meat showed no cross-reaction with any of the non-target meat. Specificity of the quadruple assay was established by carrying out the PCR.

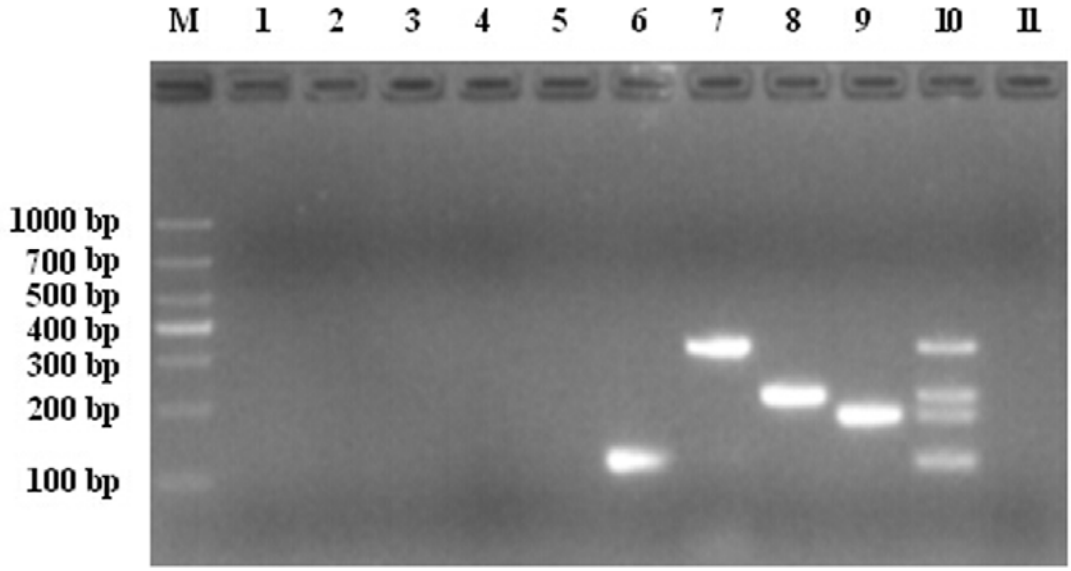

Figure 4. The specificity of the test: M, DL 1000 DNA Marker (Takara). 1, donkey meat; 2, fish; 3, shrimp; 4, mouse meat; 5, chicken; 6, beef; 7, duck; 8, pork; 9, mutton; 10, combined duck, pork, mutton and beef; 11, control reagent

3.4. The multiplex PCR Sensitivity

The results for the multiplex PCR sensitivity are shown in Figure 5. Amplification of 100 ng of meat DNAs dilutions of 100 to 0.001 four species clearly positive, and 0.1 four species the lower limit of detection. These results demonstrated that the assay sensitive to pork, beef, duck and mutton tested.

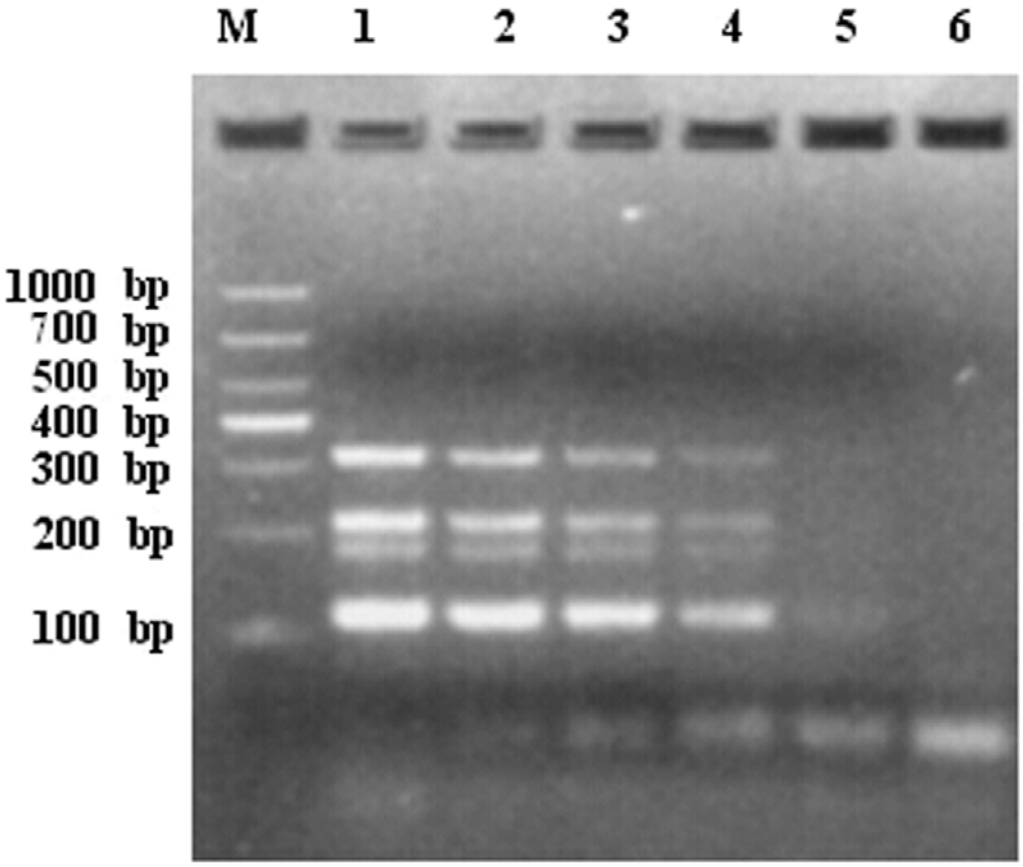

Figure 5. The multiplex PCR sensitivity. M, DL2000 DNA marker; 1-6, 100, 10, 1, 0.1, 0.01 and 0.001, respectively

4. Discussion

The aim of this study was to develop a simple method for simultaneous identification of multiple meat species. Multiplex PCR, in which many primers are used together for amplification of multiple target regions, is a hopeful technique for this purpose.

In particular, meat and meat products with higher market value are very prone to adulteration. However, the amount of "banned" products would be lowed, therefore, our study was to develop a rapid, sensitive, and reliable multiplex PCR procedure for duck, pig, bovine and sheep meat in processed meat products.

We designed two new primer pairs using the mitochondrial ND2 (NADH dehydrogenase subunit 2 genes in sheep and 16S rDNA gene in duck control region as target in this study. Pig and bovine-specific two primer pairs used cytochrome b gene (cyt b) of references. In this study, the primers successfully generated specific fragments of 177, 322, 212 and 116 bp length for sheep, duck, pig and bovine meat without cross dimers, and they were easily distinguished by agarose gel electrophoresis. The mitochondrial 16S rDNA gene Real-time PCR based assays have previously been reported by some researcher for the identification of animal derived material in meat mixtures by amplifying mtDNA genes as 16 S rDNA [42]. Species specific primers and TaqMan probes were designed targeting the mitochondrial ND5 and ND2 for the identification of donkey and pork species. Because the lengths of these genes are appropriate, their mutation degrees were sufficient and they had sequence databases available for many plant and animals [43].

The differentiation of animal species has proved to be difficult, particularly in samples of complex composition when subjected to heat. Although DNA is degraded to smaller fragments at high temperatures, amplification of target was still possible even in the autoclaved (103.4 kPa pressure, 121°C temperature for 30 min) samples. Found in line with the established fact that the heat stability and large copy number of mitochondrial DNA in meat tissue contribute to the protection and survivability of the fragments of DNA that were sufficient enough to be amplified by PCR [41]. In this research, presence of target DNA was successfully identified for each species tested, and amplification was affected by spice addition or the cooking process. Similar results were found in fermented sausages with the same primers [44,45]. Result universal primers (mcb398/mcb869) did not amplify from ham sausage (beef), canned beef and canned pork. On account of these were including large quantities of salts and preservatives. In this environment of living cells, which genome was substantial degradation. variations of DNA fragment length were observed depending on the material under examination: raw meat samples showed, in general,

intense smears of short and long DNA fragments, ranging from 2000 to10,000 bp, while DNA from Alheira samples and some tested food ingredients (such as bread and some spices) was visibly more degraded, showing sheared DNA fragments of shorter length [46].

The sensitivity of single PCR assays for each of the four primers tended to be greater when compared with the Multiplex PCR. The main reasons for this disparity were that compromises in annealing temperatures had to be made in order to accommodate the use of four primer sets for Multiplex PCR [47,48]. Nevertheless, after optimization, the multiplex PCR was capable of the simultaneous identification of all four species in a single reaction. The number of reaction cycles can affect balanced amplification of different targets, but spurious bands can occur if more than 35 cycles are used [49]. Annealing times from 90 s to 3 min and a temperature of 60 °C are best suited for amplification [50]. The optimum conditions selected in the current study were the number of reaction 40 cycles, the annealing times 45 s and temperature of 57°C best suited for amplification. The DNA template can be used 0.1ng for the Multiplex PCR.

The Multiplex PCR was used to improve amplification conditions. The specificity of the primers were tested for five meat: donkey meat, fish, shrimp, mouse meat and chicken. It was tested that primers specific to the meat showed no cross-reaction with any of the non-target meat. Specificity of the quadruple assay was established by carrying out the PCR.

5. Conclusion

This study has shown that Multiplex PCR method in meat identification and screening could be a rapid, cost-effective and easy to perform with being highly specific, reproducible and rapid (less than one working day). It should also be possible to develop additional the Multiplex PCR assays for the detection of a wider range of species used by the food industry.

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References

[1] P.D. Brodmann, D. Moor, Sensitive and semi-quantitative TaqMan real-time polymerase chain reaction systems for the detection of beef (Bos taurus) and the detection of the family Mammalia in food and feed. Meat Science, 65(1), 599-607, 2003.

[2] WANG Jian-chang, WANG Jin-feng, CHEN Rui-chun, TIAN Zhen-xiang, Molecular Biological Detection of Adulteration of Mutton and Beef with Duck. MEAT RESEARCH, 26(6), 20-23, 2012.

[3] M.E. Ali, U. Hashim, S. Mustafa, Y.B.C. Man, T.S. Dhahi, M. Kashif, M.K. Uddin, S.B.A. Hamid, Analysis of pork adulteration in commercial meatballs targeting porcine-specific mitochondrial cytochrome b gene by TaqMan probe real-time polymerase chain reaction. Meat Science, 91 454-459, 2012. (Chinese).

[4] XU Yuan-Cong , DONG Kai , HUANG Kun-Lun , XU Wen-Tao, PCR on Adulteration Detection of Pork, Beef and Mutton. Journal of Agricultural Biotechnology, 21(12), 1504-1508, 2013. (Chinese).

[5] J.J. Dooley, K.E. Paine, S.D. Garrett, H.M. Brown, Detection of meat species using TaqMan real-time PCR assays. Meat Science, 68, 431-438, 2004.

[6] M.A. Rodriguez, T. Garcia, I. Gonzalez, P.E. Hernandez, R. Martin, TaqMan real-time PCR for the detection and quantitation of pork in meat mixtures. Meat Science, 70, 113-120, 2005.

[7] M. Montowska, E. Pospiech, Species-specific expression of various proteins in meat tissue: proteomic analysis of raw and cooked meat and meat products made from beef, pork and selected poultry species. Food Chemistry, 136, 1461-1469, 2013.

[8] J.H. Calvo, P. Zaragoza, R. Osta, Technical note: A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. Journal of Animal Science, 79, 2108-2112, 2001.

[9] P.S. Girish, A.S. Anjaneyulu, K.N. Viswas, M. Anand, N. Rajkumar, B.M. Shivakumar, S. Bhaskar, Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Science, 66, 551-556, 2004.

[10] P. Ulca, H. Balta, I. Cagin, H.Z. Senyuva, Meat species identification and Halal authentication using PCR analysis of raw and cooked traditional Turkish foods. Meat Science, 94, 280-284, 2013.

[11] F. Bellagamba, F. Valfre, S. Panseri, V.M. Moretti, Polymerase chain reaction-based analysis to detect terrestrial animal protein in fish meal. J Food Prot, 66 , 682-685, 2003.

[12] A. Arslan, O.I. Ilhak, M. Calicioglu, Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat Science, 72, 326-330, 2006.

[13] S. Manzano, B. Bailey, J.B. Girodias, J. Cousineau, E. Delvin, A. Gervaix, Comparison of procalcitonin measurement by a semi-quantitative method and an ultra-sensitive quantitative method in a pediatric emergency department. Clinical Biochemistry, 42, 1557-1560, 2009.

[14] J.A. Loo, Statistics of Chi-square: X^2. Journal of Statistics, 63, 2488-2499, 1991.

[15] I. Pfeiffer, J. Burger, B. Brenig, Diagnostic polymorphisms in the mitochondrial cytochrome b gene allow discrimination between cattle, sheep, goat, roe buck and deer by PCR-RFLP. BMC Genetics, 5(30), 1-5, 2004.

[16] C. Murugaiah, Z.M. Noor, M. Mastakim, L.M. Bilung, J. Selamat, S. Radu, Meat species identification and Halal authentication analysis using mitochondrial DNA. Meat Science, 83, 57-61, 2009.

[17] P.S. Girish, A.S. Anjaneyulu, K.N. Viswas, B.M. Shivakumar, M. Anand, M. Patel, B. Sharma, Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Science, 70, 107-112, 2005.

[18] A. Macedo-Silva, S.F. Barbosa, M.G. Alkmin, A.J. Vaz, M. Shimokomaki, A. Tenuta-Filho, Hamburger meat identification by dot-ELISA. Meat Science, 56, 189-192, 2000.

[19] M. Safdar, M.F. Abasiyanik, Simultaneous identification of pork and poultry origins in pet foods by a quick multiplex real-time PCR assay using EvaGreen florescence dye. Appl Biochem Biotechnol, 171, 1855-1864, 2013.

[20] L. Cuttell, H. Owen, A.E. Lew-Tabor, R.J. Traub, Bovine cysticercosis--development of a real-time PCR to enhance classification of suspect cysts identified at meat inspection. Veterinary Parasitology, 194, 65-69, 2013.

[21] S. Soares, J.S. Amaral, M.B. Oliveira, I. Mafra, A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. Meat Science, 94, 115-120, 2013.

[22] J.J. Dooley, K.E. Paine, S.D. Garrett, H.M. Brown, Detection of meat species using TaqMan real-time PCR assays. Meat Science, 68, 431-438, 2004.

[23] F. Bellagamba, S. Comincini, L. Ferretti, F. Valfre, V.M. Moretti, Application of quantitative real-time PCR in the detection of prion-protein gene species-specific DNA sequences in animal meals and feedstuffs. J Food Prot, 69, 891-896, 2006.

[24] M. Rojas, I. Gonzalez, M.A. Pavon, N. Pegels, A. Lago, P.E. Hernandez, T. Garcia, R. Martin, Novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of meat and commercial meat products from game birds. Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 27, 749-763, 2010.

[25] Z. Kesmen, A.E. Yetiman, F. Sahin, H. Yetim, Detection of chicken and turkey meat in meat mixtures by using real-time PCR assays. Journal of Food Science, 77, 167-173, 2012.

[26] A. Rodriguez, M. Rodriguez, M.I. Luque, A. Martin, J.J. Cordoba, Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. Food Microbiology, 31, 89-99, 2012.

[27] A. Abdulmawjood, N. Grabowski, S. Fohler, S. Kittler, H. Nagengast, G. Klein, Development of Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid and Sensitive Identification of Ostrich Meat. PLoS One, 9(6), e100717. 2014.

[28] T. Kitpipit, K. Sittichan, P. Thanakiatkrai, Direct-multiplex PCR assay for meat species identification in food products. Food Chemistry, 163, 77-82, 2014.

[29] A. Dalmasso, E. Fontanella, P. Piatti, T. Civera, S. Rosati, M.T. Bottero, A multiplex PCR assay for the identification of animal species in feedstuffs. Mol Cell Probes, 18, 81-87, 2004.

[30] I. Martin, T. Garcia, V. Fajardo, I. Lopez-Calleja, P.E. Hernandez, I. Gonzalez, R. Martin, Species-specific PCR for the identification of ruminant species in feedstuffs. Meat Science, 75, 120-127, 2007.

[31] A. Dalmasso, E. Fontanella, P. Piatti, T. Civera, S. Rosati, M.T. Bottero, A multiplex PCR assay for the identification of animal species in feedstuffs. Mol Cell Probes, 18, 81-87, 2004.

[32] A. Abdulmawjood, C. Krischek, M. Wicke, G. Klein, Determination of pig sex in meat and meat products using multiplex real time-PCR. Meat Science, 91, 272-276, 2012.

[33] Novel universal primers establish identity of an enormous number of animal species for forensic application. Molecular Ecology Notes, 3, 28-31, 2003.

[34] J.J. Dooley, K.E. Paine, S.D. Garrett, H.M. Brown, Detection of meat species using TaqMan real-time PCR assays. Meat Science, 68, 431-438, 2004.

[35] T. Matsunaga, K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada, Y. Shinmura, A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science, 51, 143-148, 1999.

[36] K.F. Ebbehoj, P.D. Thomsen, Species differentiation of heated meat products by DNA hybridization. Meat Science, 30, 221-234, 1991.

[37] K.F. Ebbehoj, P.D. Thomsen, Differentiation of closely related species by DNA hybridization. Meat Science, 30, 359-366, 1991.

[38] GAO Dan- dan, CHEN Yan, WANG Ying- hua, CAO Yu- sheng, Extraction and purification of genomic DNA in the animal origin food. FOOD SCIENCE AND TECHNOLOGY, 8, 42-44, 2007. (Chinese).

[39] O. Aslan, R.M. Hamill, T. Sweeney, W. Reardon, A.M. Mullen, Integrity of nuclear genomic deoxyribonucleic acid in cooked meat: Implications for food traceability. Journal of Animal Science, 87, 57-61, 2009.

[40] T. Matsunaga, K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada, Y. Shinmura, A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science, 51, 143-148, 1999.

[41] A. Arslan, O.I. Ilhak, M. Calicioglu, Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat Science, 72, 326-330, 2006.

[42] Z. Kesmen, A. Gulluce, F. Sahin, H. Yetim, Identification of meat species by TaqMan-based real-time PCR assay. Meat Science, 82, 444-449, 2009.

[43] B.L. Herman, Determination of the animal origin of raw food by species-specific PCR. Journal of Dairy Research, 68, 429-436, 2001.

[44] Z. Kesmen, F. Sahin, H. Yetim, PCR assay for the identification of animal species in cooked sausages. Meat Science, 77, 649-653, 2007.

[45] N.S. Karabasanavar, S.P. Singh, D. Kumar, S.N. Shebannavar, Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. Food Chemistry, 145, 530-534, 2014.

[46] J.S. Amaral, C.G. Santos, V.S. Melo, M.B.P.P. Oliveira, I. Mafra, Authentication of a traditional game meat sausage (Alheira) by species-specific PCR assays to detect hare, rabbit, red deer, pork and cow meats. Food Research International, 140-145, 2014.

[47] X. Zhang, Z. Liu, J. Yang, Z. Chen, G. Guan, Q. Ren, A. Liu, J. Luo, H. Yin, Y. Li, Multiplex PCR for diagnosis of Theileria uilenbergi, Theileria luwenshuni, and Theileria ovis in small ruminants. Parasitology Research, 113, 527-531, 2014.

[48] G. Rastogi, M.S. Dharne, S. Walujkar, A. Kumar, M.S. Patole, Y.S. Shouche, Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. Meat Science, 76, 666-674, 2007.

[49] M. Weissenberger, W. Reichert, R. Mattern, A Multiplex PCR assay to differentiate between dog and red fox. Forensic Sci Int Genet, 5, 411-414, 2011.

[50] D. Sint, L. Raso, M. Traugott, Advances in multiplex PCR: balancing primer efficiencies and improving detection success. Methods in Ecology and Evolution, 3, 898-905, 2012.