APPLICATION OF PCR AND SYBR GREEN QPCR ASSAYS FOR THE IDENTIFICATION AND QUANTIFICATION OF CHICKEN MEAT UNDER DIFFERENT COOKING CONDITIONS

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Abstract – Conventional and SYBR Green qPCR assays using primers targeting the *12S rRNA* of chicken mitochondrial genes were employed for the detection and quantification of chicken meat used in food stuffs. The assays were recruited to amplify different known concentrations of DNA (0.01, 0.1, 1 ng) in the mixtures. Dissimilar kinds of processed meats were experimentally prepared using various amounts of chicken meat which were finally heated at different temperatures in order to chase up the chicken meat in the mixtures. The PCR amplification of 0.01, 0.1, 1 ng DNA revealed that the assay may amplify the species-specific amplicons as little as 0.01 ng concentration. Analysis of the experimental meat mixtures revealed the usefulness of the assays in detecting and quantifying chicken mitochondrial DNA in the mixtures.

Key Words – Chicken meat. SYBR Green qPCR. *12S rRNA*.

I. INTRODUCTION

The identification of the species of origin of meat represents a considerable problem for the food quality and food safety. Another problems need to be considered are religious issues, allergic reactions and also economic issues arising from consumption of some special meats [1-3].

Considering the above mentioned reasons, different chemical, sensory, anatomical, histological, immunological and molecular based techniques have been employed. Therefore, the need of using simple, reliable and rapid methods has been emphasized by number of researchers for determining the species of meat in meat mixed products, especially in cooked-meat preparations [4].

Molecular techniques have successfully used for the identification of different species-specific genomes. However, one need to consider that identifying the species-specific proteins is not of great helps which basically referred to the fragility of protein structures. Therefore, we have focused on using mitochondrial genomes to identify different meats mixtures and also in cooked meat products [5]. Most strategies to date have targeted mitochondrial DNA sequence because of their high copy number in animal tissues and inherent variability [6].

In the present study, we used PCR which optimized by using species-specific primers targeting the chicken meat in raw products and heated sausages. A real time PCR was additionnally used to quantify different percentages of chicken meat in the experimentally prepared sausages.

II. MATERIALS AND METHODS

Experimental design

In this study, the ratio of 1:1 of raw minced lamb and beef meat were mixed before 0.0%, 10%, 50%, and 90% of chicken meat being added to the mixture. The mixture was then uniformly homogenized and heated at pasteurization (70 ºC for 60 min), boiling (94 ºC for 30 min), sterilization (121 ºC for 20 min), and frying (160 ºC for 5min) temperatures.

DNA extraction

The specimens were initially chopped using the sterilized blender. One gram of the preparation was then homogenized in 9 ml of the normal saline. DNA extraction was carried out using the phenol-chloroform technique. The concentration of DNA was subsequently estimated by absorbance at 260 nm [7] and purity of DNA was checked by taking ratio of O.D. reading at 260 nm and 280 nm using spectrophotometer [8].

Specific primer design
Species- specific primers for the detection of chicken DNA were designed from mitochondrial genome, following the alignment of available sequences from Gen Bank (www.ncbi.nlm.nih.gov) [7].

PCR primers specificity and sensitivity test
The specificity of primers was confirmed by amplification of 100 ng purified chicken DNA/µl as positive and DNA free water as negative control. To find out the limit determination of the specific primers, different dilutions of DNA (1, 0.1, 0.01 ng DNA/µl water) were employed. The *inv* A genome of *Salmonella* from broiler was also amplified simultaneously to test the condition (Data not shown). Each dilution was used as templates in the PCR reaction mixtures [9].

Polymerase chain reaction (PCR)
PCR was carried out on 2 µl of DNA template in a final reaction mixture of 25 µl containing 2.5 µl of 10x PCR buffer, 3 mM $MgCl_2$, 200 mM of each of dNTPs, 400 µM of each of forward and reverse primer, 2 units of Taq DNA polymerase (Cinagene, Iran). The PCR cycling was performed in a gradient thermocycler (Eppendorf, Germany) with an initial denaturation step at 94 ºC for 2 min followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 62 ºC for 1 min and extension at 72 ºC for 1 min . Then final extension was done at 72 ºC for 5 min (BIOR XP, China). The amplified products were subsequently electrophoresed in 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A species specific fragment of 450 bp was then amplified corresponding to the *12S rRNA* genome of chicken.

SYBR Green real time PCR assay
This method used for the detection and quantification of chicken DNA in meat product. It is based on the DNA fragments in mitochondrial gene. In this study, all the reactions were set up using SYBR Green that stain joint to double strand DNA and fluorescent wave was realized until absorption with acceptor molecule. Final volume was 20µl containing 4 µl DNA, 2 µl primers, 14 µl H_2O. The conditions for thermal cycling were set according to the PCR process. Continuous monitoring of fluorescent signal was carried out at the last step. During this period, a rapid decrease in the fluorescence occurs due to the denaturation of the amplicons, such that single strand of DNA appear with the successive detachment of the SYBER-Green, as was previously described by Martin et al. [10].

III. RESULTS AND DISCUSSION

The primers designed from specific fragments (450 bp) of chicken DNA. The specificity of primers was confirmed by gene sequencing.

Our results indicated that cooking methods showed minor effects on the amplification of the target genomes (Figures 1 and 2).

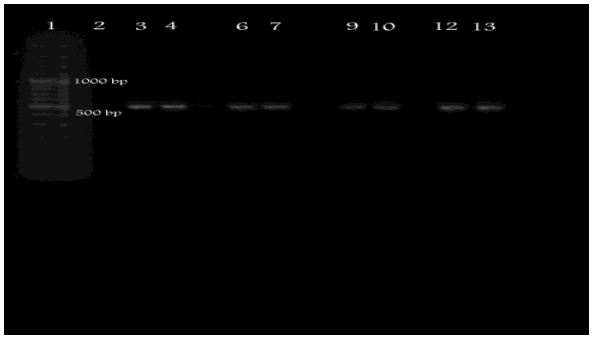

Figure 1 Detection of 450 bp *12S rRNA* in meat prepations: comparison between different DNA concentrations extracted from various proportions of the specimen using conventional PCR (Lane 1: 100 bp Marker, Lane 2 negative control (no template), Lane 3 & 4 pasteurized meat mixtures at 10 % and 50% proportions , Lane 6 & 7 boiled meat mixtures at 10 % and 50% proportions, Lane 9 & 10 sterilized meat mixtures at 10 % and 50% proportions, Lane 12 & 13 fried meat mixtures at 10 % and 50% proportions.

The mitochondrial gene encoding the *12S rRNA* was chosen as a target for chicken DNA quantification [11]. As shown in Figure 3, different concentrations of raw chicken meat were detected, based on the threshold cycle (Ct) of each sample which is, the cycle number where the samples fluorescent curve sharply upward and corresponds to the initial

concentration of DNA. In general, the higher the Ct, the lower the initial concentration of DNA. The technique was robust to detect 0 to 90 % ratios of the chicken materials in experimentally made sausages. Further to investigate whether different temperatures may cause DNA degradation in the food stuffs, here, we have employed various ranges of thermal conditions and as it has clearly demonstrated no dramatic influence was shown on the amplification of DNA fragments (Figure 4).

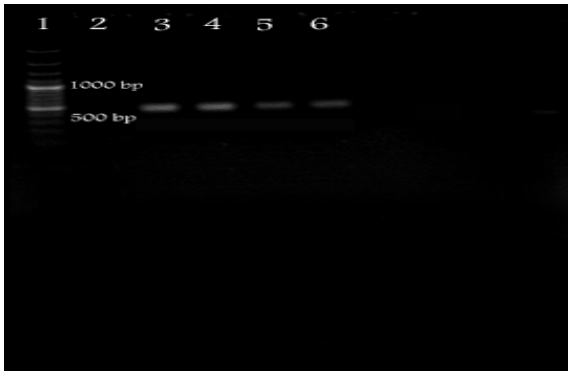

Figure 2 Detection of 450 bp *12S rRNA* in meat prepations: comparison between different DNA concentrations extracted from various proportions of the specimen using conventional PCR (Lane 1: 100 bp Marker, Lane 2 negative control (no template), Lane 3 pasteurized prepared meat mixtures at 90% proportions, Lane 4 boiled prepared meat mixtures at 90 % proportions, Lane 5 sterilized prepared meat mixtures at 90 % proportions, Lane 6 fried prepared meat mixtures at 90 % proportions.

The aim of the present study was to develop and evaluate a species-specific PCR assay for identification of chicken species in meat under different thermal conditions. This is one of the valuable tools to identify the chicken materials in the presence of other meat species DNA [6].

The species-specific primer was designed based on the mitochondrial D-loop gene for amplification of the 450 bp DNA fragments from sequences available in the Gen bank database for chicken DNA. The mitochondrial DNA was targeted to design the primers, because of inherent multi copy presence and great sequence diversity of the mitochondrial genes [12]. Other workers also suggested that mitochondrial markers are more efficient than nuclear markers for the purpose of identification and authentication of meat species [6].

Our assay was exceedingly sensitive and detected the presence of 0.01 ng of template DNA when assessed using different dilutions of DNA which was also supported by Kesman et al. [9].

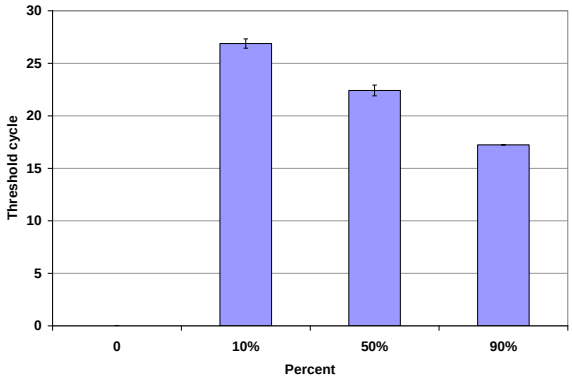

Figure 3 Quantification profiles of different concentration of chicken meat in experimentally prepared food stuffs.

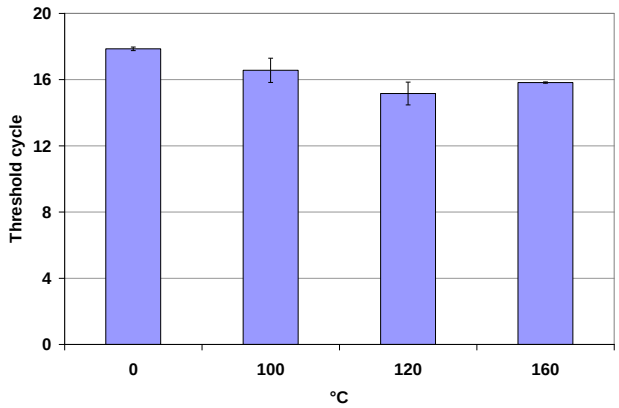

Figure 4 Quantification profiles of the highest (90%) concentration of chicken meat in experimentally prepared food stuffs-effect of different temperatures conditions.

In the current work, the PCR was optimized in order to detect the genome in both raw and cooked (prepared under four conditions) meat emulsions. As mentioned earlier, this may be due to the heat stability and also large number copies of mitochondrial DNA in meat tissue contributing to the stability of the DNA copies [8]. Earlier, Hird et al. [13] were successfully applied chicken and turkey specific primer pairs

based on mitochondrial cytochrome *b* gene for the amplification of template DNA isolated from raw, boiled and autoclaved chicken and turkey meat.

These results clearly revealed that species-specific PCR method is sensitive, specific, convenient and cheap assay for rapid identification of very low percentage of chicken meat in meat adulteration, even in meat mixture subjected to different processing condition. The application of the Q-PCR on the detection of targeted amplicon following the use of different thermal conditions was also approved, which was more likely due to the stability and high copy number of mitochondrial sequences.

IV. CONCLUSION

The SYBR-Green real-time PCR system provided an applicable technique to detect very low concentrations of chicken materials in food products and thus could be proposed as a reliable technique to prevent frauds in the meat industries. Moreover, as the sterilization and/or frying temperature were not influencing the extracted DNA, the technique is strongly advised for the routine analysis of cooked chicken meat, as well.

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REFERENCES

1. Asensio, L., González, I., García, T. & Martín, R. (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control 19: 1-8.
2. Bai, W., Xu, W., Huang, K., Yuan, Y., Cao, S. & Luo, Y. (2009). A novel common primer multiplex PCR (CP-M-PCR) method for the simultaneous detection of meat species. Food Control 20: 366–370.
3. Stamoulis, P., Stamatis, C., Sarafidou, T. & Mamuris, Z. (2010). Development and application of molecular markers for poultry meat identification in food chain. Food Control 21: 1061-1065.
4. Sawyer, J., Wood, C., Shanahan, D., Gout, S. & McDowell, D. (2003). Real-time PCR quantitative meat species testing. Food Control 14: 579–583.
5. Martın, I., Garcıa, T., Fajardo, V., Lopez-Calleja, I., Rojas, M., Hernandez, P. E, Gonzalez I. & Martin, R. (2007). Mitochondrial markers for the detection of four duck species and the specific identification of Muscovy duck in meat mixtures using the polymerase chain reaction. Meat Science 76: 721–729.
6. Hopewood, A. J., Fairbrother, K, S., Lockley, A.K. & Bardsley, R. G. (1999). An action gene-related polymerase chain reaction (PCR) test for identification of chicken in meat mixtures. Meat Science 53: 227-231.
7. Kesmen, Z. A. (2005). Research on the meat species identification in fresh and processed meat products by using PCR techniques. Ph.D. Dissertation, Ataturk University, Erzurum.
8. Haunshi, S., Basumatary, R., Grish, P. S., Doley, S., Bardoloi, R. K. & Kumar, A. (2009). Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. Meat Science 83: 454-459.
9. Kesman, Z., Sahin, F. & Yetim, H. (2007). PCR assay for the identification of animal species in cooked sausages. Meat Science 77: 649-653.
10. Martin, I., Garcia, T., Fajardo, V., Rojas, M., Pegels, N. & Hernandez, P. H. (2009). SYBR-Green real-time PCR approach for the detection and quantification of pig DNA in feed stuffs. Meat Science 82: 252-259.
11. Grish P. S., Anjaneyulu A. S. R., Viswas K. N., Shivakumar B. M., Anand, M., Patel, M. & Sharma, B. (2005). Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Science 70: 107-112.
12. Brown, G., Gadaleta, G., Pepe, G., Saccone, C. & Sbisà, E. (1986). Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. Journal of Molecular Biology 192: 503-511.
13. Hird, H., Goodier, R. & Hill, M. (2003). Rapid detection of chicken and turkey in heated meat products using the polymerase chain reaction followed by amplicon visuzlisation with vistra green. Meat Science 65: 1117-1123.