International Food Research Journal 25(2): 632- 637(April 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Real-time polymerase chain reaction (RT-PCR) for the authentication of raw meats

Chung, H.H

Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

Article history

Received: 6 January 2017
Received in revised form: 2 February 2017
Accepted: 4 February 2017

Keywords

RT-PCR, D-loop
SYBR Green
Raw meat
Species-specific primers

Abstract

Meat adulteration has been a significant issue in today's food industry as it intertwine with religious, social and economic values. PCR based techniques for the detection of meat species in a meat admixture are primarily used by the industry as a reliable approach due to its sensitivity and reliability. This paper describes the design and verification of real-time polymerase chain reaction (RT-PCR) based assay for the detection of meat from various non-target species by using species specific oligonucleotides. Five sets of species-specific primers have been developed to target small regions (≤ 150 bp) of the mitochondrial D-loop. The specificity, sensitivity and reliability of each assay have been verified by using SYBR Green based RT-PCR. By using a cut-off CT of 30 cycles, all assays show sensitivity down to 0.05% of the DNA spike level. When applied to DNA templates from raw meat admixtures, assays were able to detect the target species up to a level of 0.1%. Hence, this verify the potential applicability of these assays in the meat industry.

Introduction

Currently, several techniques are available for meat species identification. These are based primarily on analysis of the biochemical component of meat. They include protein based methods (i.e. high performance liquid chromatography (Espinoza *et al.*, 1996), electrophoretic methods (Mansfield *et al*., 1998) and immunological methods (Macedo-Silva *et al.*, 2000; Hajmeer *et al*., 2003), nucleic acid analysis, fatty acid analysis, determination of microscopic structured elements (Boyaci *et al*., 2014) spectroscopy using visible and infra-red wavelength (Rannou and Downey, 1997) and measurement of isotope abundancy (Ghidini *et al*., 2006). Although useful under ideal conditions, most of these methods have major drawbacks. For example, the protein and immunological based techniques become insensitive when applied to heat treated meat due to protein denaturation. Fatty acid determination serves fairly well but it rather has limited application in the food industry due to lipid oxidation during food processing.

Nucleic acid based authentication techniques currently available include the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Lanzilao *et al*., 2005), slot blot hybridization, single strand conformational polymorphism (SSCP) (Rehbein *et al.*, 1997), DNA-DNA hybridization (Chikuni *et al*., 1990), DNA sequencing (Bartlett and Davidson, 1992) and MIR-PCR (Buntjer and Lenstra, 1998). Of all, PCR-RFLP has been evaluated for its efficiency in the identification of both cooked and uncooked tissues, although the method is unsuitable for analysing meat mixtures (Partis *et al*., 2000). The recent advent of real-time polymerase chain reaction technique has facilitated the specific identification of meat samples as well as the ability to detect minute amounts of target species in a DNA admixture (Lopez-Andreo *et al*., 2005; Rodriguez *et al.*, 2005; Fajardo *et al*., 2008). PCR analysis of species specific mitochondrial DNA sequences has been widely used for the identification of meat species. Sequences of the mtDNA which have been frequently used for meat analysis include the 12S rRNA (Fajardo *et al*., 2008), cytochrome b (Branicki *et al*., 2003; Dooley *et al*., 2004), 16S rRNA (Guha and Kashyap, 2005; Mane *et al*., 2013) and the D-loop region (Sawyer *et al*., 2003).

The aim of this study is to design new primer sets to allow sensitive and reliable authentication of several economically important meat species such as pork *(Sus scrofa),* cattle *(Bos taurus),* buffalo *(Bubalus bubalis)*, chicken *(Gallus gallus)* and turkey *(Meleagris gallopavo)* based on real-time polymerase chain reaction (PCR). Successful application of molecular techniques in the authentication of meat

*Corresponding author.
Email: *hhchung@unimas.my*

products plays an important role in establishing the halal-based industry with wide economic implications.

Materials and Methods

Design of specific primers

Gene sequences for the D-loop region of meat species were recovered from the NCBI GenBank. The accession number for the meat species were AF034253 *(Sus scrofa)*, NC006853 *(Bos Taurus),* NC005044 *(Capra hircus)*, AF475278 *(Bubalus bubalis)*, DQ903207 *(Ovis aries),* NC001323 *(Gallus gallus)* and NC010195 *(Meleagris gallopavo).* Sequence alignment was performed using the CLUSTAL W (Thompson *et al.*, 1994) sequence alignment software available online at the SDSC Biology Workbench website *(http://workbench.sdsc.edu/)*. Regions of sequence variability were selected for species specific primer design. Primers and probes were designed using online software Primer3 *(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)*, tested for self-complimentarity using the OligoCalc *(http://www.basic.northwestern.edu/biotools/oligocalc.html)*, and synthesized.

Prior to the design of probes, primers pairs were tested for their specificity by performing hot start, end-point PCR with GoTaq Flexi DNA polymerase (PROMEGA), in a total volume of 20 µl containing 10 pmol of each primer, 50 ng of DNA template and 2.5 mM $MgCl_2$. Amplification was performed in a Mastercycler personal 5332 (Eppendorf) according to the following PCR step-cycle program: initial denaturation and enzyme activation at 95°C for 5 mins followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. Product amplification was confirmed using standard agarose gel electrophoresis techniques.

DNA admixtures preparation from various meat kinds

Total DNA was extracted from each of the seven meat samples using the DNeasy DNA isolation kit, according to the manufacturer's instructions (Qiagen), and quantitated spectrophotometrically at a wavelength of 260 nm using CaryWin UV spectrophotometer (Varian, Zug, Switzerland). Stock DNA was then diluted to obtain 50 ng/µl DNA preparations for each species. DNA admixtures were prepared by mixing DNA solution from different meat species to obtain preparation with 1%, 0.5%, 0.25%, 0.1%, 0.05%, 0.025% and 0.01% of relative concentrations. Total DNA from 2g of meat samples was extracted from raw meat admixture through CTAB extraction method as described by (Dooley *et al*., 2004). The presence of DNA in the samples was then confirmed by amplification using 18S rRNA universal primers: EUKF- 5'-AGCCTGCGGCTTAATTTGAC-3' and EUKR- 5'-CAACTAAGAACGGCCATGCA-3' (Lopez-Andreo *et al*., 2005).

Development of real-time PCR assay

The real time PCR assay was carried out on the Biorad iCycler IQ multicolor RT-PCR detection system in conjunction with the SYBR Green detection chemistry which included: iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, 0.4 mM of each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers). Reactions were carried out in a final volume of 50 µl containing 1 µl of meat DNA template (50 ng), 300 nM of each primer, and 25 µl of iQ SYBR Green Supermix for 10 min at 95°C, 40 cycles of 40 s at 95°C, 20 s at 55-65°C (depending on the annealing temperature of primer used), and 30 s at 72°C.

Verification of real-time data obtained

Standard curves for each assay was drawn according to the equation outlined by (Higuchi *et al*., 1993). Efficiencies and sensitivities of these assays were evaluated according to the standard curves derived from the equation. The detection limit and reliability was also evaluated by using the equation outlined by (Lopez-Andreo *et al.*, 2005).

Results and Discussion

Species specific primer pairs

Primers were designed to flank the regions of the D-loop for various meat samples. These were done by aligning all the D-loop sequence from different animal species by using CLUSTALW sequence alignment software. The alignment of the sequences showed no region of similarity for all the species tested. These observations indicate that no single universal probe could be designed for all the species (data not shown). Subsequently, single pair of primer was selected for final assay development for each species by using end point PCR. Primers were selected to show no primer-dimerization and cross reactivity with DNA from other species, as determined in silico by using the BLASTn suite (data not shown).

The primer pairs that were selected for assay development are listed in Table 1. The length of the primers ranged from 18 bp to 23 bp, and they produced amplicons with sizes ranging from 131 bp to 150 bp. Each primer had a different annealing temperature

according to its G:C content. To permit amplification of DNA to occur, an annealing temperature for each set of assays involving both the forward and reverse primer was determined. These temperatures were in the range of 50-60°C. The conditions were then applied to real-time PCR using iQ SYBR Green Supermix with slight modification in template DNA concentration, which was reduced from 150 ng to 50 ng for the real-time detection system. Of all the primers and probes designed for seven animal species, only five sets were confirmed for their specificity. These were SSDL *(Sus scrofa),* GGDL *(Gallus gallus)*, BTDL *(Bos taurus)*, BBDL *(Bubalus bubalis)* and (MGDL) Meleagris gallopavo. The specificity of these primers was evaluated by using end point PCR prior to the real time application.

Efficiency, specificity and detection limit of the PCR relations

The amount of template suitable for performing real-time analysis was established at 50 ng which yielded CT values in the range of 16 to 18 cycles. CT value refers to the PCR cycle at which fluorescence is detected above the background fluorescence. The CT values obtained in this study ranged from 16.4 to 17.2. It is important to note that real time PCR data obtained by using different primer pairs are only comparable when the PCR efficiencies for the different reactions are similar. The theoretical optimal efficiency should approach a value of 2 which represents doubling in the number of product copies number (Lopez-Andreo *et al.*, 2005). The efficiencies of each assay were estimated from a standard curve based on serial dilution of a standard that carries the target sequence (Rutledge and Cote, 2003). The C_T values of the diluted standards were read out, and plotted versus the logarithm of the samples' concentrations which conform to the following equation (Higuchi *et al.*, 1993):

$$C_T = (1/\log E) \log(N_0) + (\log N_c/\log E)$$

where E is the efficiency, N_0 is the initial amount of DNA and N_c is the amount of DNA at C_T cycle. Hence, the PCR efficiency was calculated from the slope as 1/logE. The efficiencies of all the five set species specific primers and the 18S detectors were calculated from C_T versus log N plots using fourfold serial dilutions of 100 ng to 0.5 ng of purified DNA from each species. Dilutions resulting in template amount lower than 0.5 ng were not used to estimate the efficiency of assay as the linearity of the standard curve was often lost at this stage. The PCR efficiencies obtained for all five sets of primers ranged from 1.89 to 2.00 as indicated in Table 2.

The specificity of each assay was tested by running real time PCRs with 50 ng pure DNA from different meat types. At the initial stage of primers screening, several primer sets produce no background cross-reaction electrophoretically (on agarose gel). However, some residual fluorescent signal could be detected in real-time analysis despite having high C_T values. This may indicate the higher sensitivity of real-time analysis compared to conventional PCR. Hence to measure the cross reactivity of each assay to non-target species, a standard of 40 cycles of real time PCR was utilized. To examine the cross reactivity of each assay, the signal to noise ratio, R, was measured. This was done by using the following equation developed by (Lopez-Andreo *et al*., 2005):

$$R = E^{Cs-Cs,x}$$

whereby C^s is the resulting C_T when an assay was applied to its target species, $C^{s,x}$ is the C_T obtained when the same assay is applied to a non-target species at the same amount of template. To obtain the percentage of cross reactivity, the signal to noise ratio was multiplied by 100. Results showed little or no cross reactivity were observed for all assays as shown in Table 2. The highest cross reactivity observed was 0.008% which occurred between cattle assay with pork DNA. All assays except for turkey specific assay, showed some degree of cross-reactivity with non-target species DNA. It should be noted that a difference in 1 C_T value between the target species and non-target species represents a cross reactivity of 50% of the assay with non-target species. Theoretically, this indicates that a double amount of non-target species would be required to give the same C_T as the target species. The cross reactivity percentage is hence halved for each extra C_T obtained. Limit of detection can be calculated by using the standard curves produced for each assay. To determine the limit of detection, a cut off C_T of 30 was used. A cut off C_T represents the cut-off point for positive identification of a species. The limit of detection has been calculated for each species specific assay with turkey specific assay having the highest limit of detection, 24.6 pg. The limits for reliable detection for all other assays were lower than 10.0 pg.

Most of the assays developed in this work produced detection up to 0.05% relative to the mixer species. This corresponds to a 25 pg of target DNA in 50 ng of the non-target species. These assays were believed to reach its practical limit of detection. The limit of detection found in Table 2 is in accordance

with the theoretical limit of detection (at cut-off C_t = 30) calculated in Table 1 by using the standard curve derived from each specific assay. Through this study, it was also found that 50 ng of template DNA does not inhibit any PCR reaction which has occurred in the detection system developed by (Dooley *et al.,* 2004).

Sensitivity of primers

The sensitivity of the all the species specific primer sets were evaluated by applying it to DNA admixtures of the target species spike at a concentration of 1.0%, 0.5%, 0.25%, 0.1%, 0.05%, 0.025% and 0.01% relative to the non-target species DNA template. Referring to Table 3, it was found that positive detection was observed for all spike levels except 0.025% and 0.01%. At a cut off limit of 30 cycles, PCR results showed that even at a low relative percentage such as 0.05%, the target species could still be detected with its specific primers and assay conditions. In this study, a spike level of 0.05% would correspond to a target species DNA template concentration of 0.025 ng. The C_T values obtained at this relative percentage ranged between 26.4 to 30.5 cycles. These results indicate that these assays should have reached their lower limits of detection. This observation corroborates the lower limit of detection determined by using the standard curve obtained according to each species respectively. From Table 3, it can be observed that at a spike level of 0.01%, no positive detection was observed for the pork assay regardless of the type of non-target species DNA used. All C_T values obtained were in the range of more than 30 cycles which is in excess of the cut off C_T set for the purpose of this assay. Similar observation can be seen for other assays where most of the C_T values were close to the cut off C_T or exceeded it.

The observed C_T's for cattle assay at a detection level 0.1% relative to its non-target DNA was approximately 9 or 10 cycles higher than those observed when the assay were applied to pure beef. An expected C_T would be approximately 26.9 cycles since halving the amount of template DNA increases the C_T value by 1. In this case the difference between 50 ng and 0.05 ng (0.1% spike) is about 210, which is about 10 PCR cycles. Observed C_T values at this level of detection falls within C_T of 25.9 to 27.5, which means that the cattle specific assay can still be further used to detect cattle's DNA at a spike level lower than 0.1% relative to the non-target species.

Reliability of primers

Throughout this study, it was found that the universal primers signal which act as a control signal is often non-identical to signal produced by species specific primers even though similar amount of template were present. This observation has been explained earlier by (Lopez-Andreo *et al*., 2005) as a result of interspecies variations in the copy number of the target gene and the consensus sequence. Due to such differences, the signal produced by the consensus sequence is often not be taken as an estimate of the total DNA template present in a reaction which involves two or more DNA template from different species. Hence, to evaluate whether this method is reliable in the quantification of two or more mixed species, an alternative method of analysis has been suggested by (Lopez-Andreo *et al*., 2005) which is by measuring the ratio of theoretical and experimental C_T values of the 18S consensus sequence. As outlined, the theoretical C_T value can be calculated using the following equation:-

$$C^{c,th} = -3.3\log(NT) + b_T$$

where N_T is the total amount of template present in a reaction which can be calculated from each species specific standard curve while b_T is the intercept for the 18S theoretical standard curve. b_T can be calculated by using the equation:-

$$b_T = b^e_A + N_B(b^e_B - b^e_A)/N_T, \quad \text{if } b^e_B > b^e_A$$

where b^e_A and b^e_B are the intercepts of the 18S standard curves for each individual species. Table 4 shows an example of the rationality of these equations in measuring the reliability of cattle *(Bos taurus)* and buffalo *(Bubalus bubalis)* specific assay. By comparing the experimental C_c values with the theoretical $C^{c,thb}$ value, a ratio can be derived which reflects whether the signal produced by the consensus sequence actually matches the contribution of target sequence from each individual species. From the example given below, it can be seen that the ratios, $C^{c,thb}/C_c$ found have correctly reflected the consistencies of the 18S consensus sequence with the sum of DNA template from each individual species. This indicates that no additional DNA template were present in all the reaction mixtures, hence, the NBos and NBub values calculated in the table can be used to represent the proportion of each species DNA in the reaction mixture.

Detection of target species in raw meat admixtures

The developed assays were used to test raw meat admixtures at 5.0%, 1.0%, 0.5% and 0.1% spike level. Lower spiking level was not used in this study due to the un-uniformity of the target species in the

raw meat admixture. All analysis was carried out by adding weighted portion of lean meat tissue into each other. CTAB extraction method was used instead of using DNA isolation kit to ensure the uniformity of the analysis. Results of raw meat admixtures analysis is shown in Table 5. C_t values obtained provide a relative indication of the percentage of meat content in the admixture. The results indicate a good agreement of the amount of DNA detected and proportion of the raw meat admixture. However most the results show an overestimation of the target species percentage relative to the spiked species.

Conclusion

This study has indicated that it is possible to develop a method based on species specific real-time PCR assay. By setting a cut-off C_T at 30 cycles, the reliability and sensitivity of these assays were verified. *Sus scrofa* and *Bos taurus* specific assay were able to detect the target species with a lower limit of 0.05% relative to non-target species. The application of a pair of primers comprising one universal primer and one species specific primers in the method developed indicates that an accurate and reliable technique has been developed for meat authentication and quantification. In addition, the use of control primers also allowed comparative measurement and normalization of amplification response between unknown samples and the standard reference samples.

Acknowledgement

This work was fully funded by The Ministry of Higher Education, Malaysia through the Fundamental Research Grant Scheme FRGS/SG05(03)/1148/2014(15).

References

Bartlett, S.E. and Davidson, W.S. 1992. FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. BioTechniques 12: 408-411.

Boyaci, I.H., Temiz, H.T., Uysal, R.S., Velioglu, H.M., Yadegari, R.J. and Rishkan, M.M. 2014. A novel method for discrimination of beef and horsemeat using Raman spectroscopy. Food Chemistry 148: 37-41.

Branicki, W., Kupiec, T. and Pawlowski, R. 2003. Validation of cytochrome b sequence analysis as a method of species identification. Journal of Forensic Science 48: 83-87.

Buntjer, J.A. and Lenstra, J.A. 1998. Mammalian species identification by interspersed repeat PCR fingerprinting. Journal of Industrial Microbiology and Biotechnology 21: 121-127.

Chikuni, K., Ozutsumi, K., Koishikawa, T. and Kato, S. 1990. Species identification of cooked meats by DNA hybridization assay. Meat Science 27: 119-128.

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

Espinoza, E.O., Kirms, M.A. and Filipek, M.S. 1996. Identification and quantitation of source from hemoglobin of blood and blood mixtures by high performance liquid chromatography. Journal of Forensic Science 41: 804-811.

Fajardo, V., Gonzalez, I., Martin, I., Rojas, M., Hernandez, P.E., Garcia, T. and Martin, R. 2008. Real-time PCR for detection and quantification of red deer *(Cervus elaphus),* fallow deer *(Dama dama)*, and roe deer *(Capreolus capreolus)* in meat mixtures. Meat Science 79: 289-298.

Ghidini, S., Ianieri, A., Zanardi, E., Conter, M., Boschetti, T., Iacumin, P. and Bracchi, P. G. 2006. Stable iSotopeS determination in food authentication: a review. Ann. Fac. Medic. Vet. di Parma. XXVI:193-204.

Guha, S. and Kashyap, V.K. 2005. Development of novel heminested PCR assays based on mitochondrial 16s rRNA gene for identification of seven pecora species. BMC Genetics 6: 42.

Hajmeer, M., Cliver, D.O. and Provost, R. 2003. Spinal cord tissue detection in comminuted beef: comparison of two immunological methods. Meat Science 65: 757-763.

Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y) 11: 1026-1030.

Lanzilao, I., Burgalassi, F., Fancelli, S., Settimelli, M. and Fani, R. 2005. Polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial cytb gene from species of dairy interest. Journal of AOAC International 88: 128-135.

Lopez-Andreo, M., Lugo, L., Garrido-Pertierra, A., Prieto, M.I. and Puyet, A. 2005. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. Analytical Biochemistry 339: 73-82.

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

Mane, B.G., Mendiratta, S.K., Tiwari, A.K. and Narayan, R. 2013. Sequence analysis of mitochondrial 16S rRNA gene to identify meat species. Journal of Applied Animal Research 41: 71-81.

Mansfield, E.S., Robertson, J.M., Vainer, M., Isenberg, A.R., Frazier, R.R., Ferguson, K., Chow, S., Harris, D.W., Barker, D.L., Gill, P.D., Budowle, B. and McCord, B.R. 1998. Analysis of multiplexed short tandem repeat (STR) systems using capillary array electrophoresis. Electrophoresis 19: 101-107.

Partis, L., Croan, D., Guo, Z., Clark, R., Coldham, T. and Murby, J. 2000. Evaluation of a DNA fingerprinting

method for determining the species origin of meats. Meat Science 54: 369-376.

Rannou, H. and Downey, G. 1997. Discrimination of Raw Pork, Chicken and Turkey Meat by Spectroscopy in the Visible, Near- and Mid-infrared Ranges. Analytical Communications 34: 401-404.

Rehbein, H., Kress, G. and Schmidt, T. 1997. Application of PCR-SSCP to Species Identification of Fishery Products. Journal of the Science of Food and Agriculture 74: 35-41.

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

Rutledge, R.G. and Cote, C. 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Research 31: e93.

Sawyer, J., Wood, C., Shanahan, D., Gout, S. and McDowell, D. 2003. Real-time PCR for quantitative meat species testing. Food Control 14: 579-583.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.