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Quantitative detection of chicken meat routine mislabelling in emulsion type sausages and burgers by SYBR green real time PCR assay

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ABSTRACT- Today, the authenticity of meat products with less costly and desirable species has increased. Therefore and considering religious, economical or public health concerns, proper actions should be taken to prevent such frauds. In this study, real time PCR assay was applied for rapid, sensitive and specific identification and quantification of chicken tissue in meat products. Specific primers were designed on the 12*S rRNA* chicken mitochondrial genes. The conventional PCR and SYBR Green RT-PCR were employed on DNA extracted from 150 samples, sausages and burgers. The results showed the presence of misused chicken meat in the sausages and burgers were 84% and 26%, respectively. Additionally, the Q-PCR assay was able to detect estimate rates of 10% to 50% of chicken meat in the products. The detection limit of the method could be quantitatively used by regulation authorities to control the quality of meat products.

INTRODUCTION

Nowadays, due to the high nutritional values, varieties, longer shelf life, cheaper prices, and dramatic changes in life styles, consumption of the cooked meat products has become very popular. As such, the opportunity for fraudulent labelling has increased due to the complexity of detection. The most common fraudulent practices are substitution of lower quality animal tissues by meat in meat products along with the application of unlicensed food additives in them.

The prevalence of this practice which has raised public concern and attention is now focused on correct food labelling. This also has implications for industry reputation and fair trade.

In the past, there were insufficient methods/ regulations for effective detection and prevention of these kinds of fraudulent. To ensure adherence to regulations, and to enforce punitive measures when needed, robust analytical tests are required (Aguado et al., 2001; Ballin, 2010). For this purpose, a range of analytical techniques have recently been developed based on detecting protein or DNA molecules. However, some researchers suggest that protein based methods such as enzymatic assays, high performance liquid chromatography, and electrophoresis techniques are less sensitive and inadequate for the species identification of thermally processed foods. They are considered to be a time-consuming and costly form of analytical method. For these reasons, they now use mitochondrial DNA for the purpose of meat species identification. DNA has a higher thermal stability, which is present in the majority of cells and potentially enables identical information to be obtained from the same animal, regardless of the tissue of origin (Ballin et al., 2009).

The advantage of mitochondrial based DNA analysis derives from the fact that there are many mitochondria per cell and many mitochondrial DNA molecules within each mitochondrion, making mitochondrial DNA a naturally amplified source of genetic variation (Hopewood et al., 1999; Rojas et al., 2011). Here, we have used the *12S r*RNA as a target amp icon because of its wide use in the recent works (Matsonaga et al., 1999; Verkaar et al., 2002).

Among DNA based methods, PCR has extensively been applied for the detection of animal species in a wide range of raw and processed foods (Verkaar et al., 2002). This method is sensitive, specific, convenient, precise and rapid for suitable identification of animals' DNA (Rodriguez et al., 2005; Martin et al., 2009; Fajardo et al., 2010). Standard PCR assays allow for the qualitative detection of different animal species in a mixture, although they are not suitable for quantitative determination of the animal tissue in the product. Due to this reason, the most recent reports have focused on the use of Real time PCR for meat species identification

and quantification (Kesman et al., 2009; Stamoulis et al., 2010).

Currently, two methods of correlating PCR products with the fluorescent are available; namely, SYBR Green and TaqMan Real time. SYBR Green dyes bind to all double stranded DNA present, and its specificity only depends on two PCR primers. One advantage of this method is that a melting curve can be generated in it after PCR so that single mutation and deletions can be detected. TaqMan probes utilize an additional primer, which also binds specifically to the target DNA sequence (Ballin et al., 2009; Kesman et al., 2009).

The aim of this study was to develop and evaluate a real time PCR assay for the identification and quantification of chicken tissues in red meat products. In this regard and in order to detect mislabeling in meat product, SYBR Green RT PCR method was used for the quantification and identification of chicken meat in emulsion-type sausages and burgers.

MATERIALS AND METHODS

Selection of Meat Samples and DNA Extraction

In this study, 100 random samples of emulsion type sausages with different percentage of red meat such as 40%, 55%, 60%, 70%, 80%, 90% and 50 samples of raw burgers with different amount of red meat (30%, 60% and 90%) were collected from local market. Selected samples were stored at -20 °C until use. The specimens were initially chopped using a sterilized blender. One gram of the sample was then homogenized in 9 ml of standard saline solution. Phenol: chloroform: isoamylalcohol, a rapid and simple DNA extraction solution was used for the extraction, based on the incubation of the sample in lysis buffer containing 20% chelex to remove PCR inhibitors. The mixture was heated to 95 °C for 15 min, centrifuged at 10,000 g and added directly to the PCR reaction as was previously optimized by Wang et al. (2000), before being tested in several sausages samples (Wang et al., 2000). The supernatants were discarded before adding 250 µl of buffer 1 (resuspension solution contained 100 µg/ml RNase) and 250 µl of buffer 2 (Lysis buffer), 550 µl saturated phenol was then added, mixed thoroughly and centrifuged again at 8,000 g for 5 min. The supernatant was collected into a new eppendorff; the same volume of the phenol was added and centrifuged at the same speed. The clear phase was collected into a new tube, before adding sodium acetate (2M, pH~5.2). The aliquot was mixed with 1.5 ml 100% ethanol, kept at -20 ºC for 1 hour, centrifuged at 12,000 g, the supernatant was then discarded and the DNA pellet was washed with 80% ethanol, before being dried and resuspended in 30 µl TAE until further use. The concentration of DNA was subsequently estimated by absorbance at 260 nm and purity of DNA was checked by taking the ratio of O.D. reading at 260 nm and 280 nm using spectrophotometer (Kesman, 2005; Hanushi et al., 2009).

Specific Primer Design

Species-specific primers for the detection of chicken DNA were designed from 12*S rRNA* mitochondrial genome, following the alignment of available sequences from Gen Bank (www.ncbi.nlm.nih.gov) (Kesman, 2005). The primer pairs designed were prepared by Gene Fanavaran, Tehran, Iran. Meat species identification of chicken was further validated by checking it for cross amplification in other species such as cattle, sheep, goat, pig, rabbit, duck, pigeon and turkey (Hanushi et al., 2009). Details of the primer pair used in the present investigation are given below:

Forward 5' – ctcgccctacttgccttc c – 3'
Reverse 5' – tag gacgcaacg cag gtg - 3'

PCR Primers Specificity and Sensitivity Test

The specificity of primers was confirmed by amplification of 100 ng purified chicken DNA/µl as the positive control and DNA free water as the 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. Each dilution was used as template in the PCR reaction mixtures (Kesman et al., 2007).

SYBR Green Real Time PCR Assay

The SYBR Green real time PCR assay method is used for the detection and quantification of chicken DNA in meat product. It is based on the species-specific fragmentation of 450 bp which was then amplified corresponding to the 12*S rRNA* genome of chicken mitochondrial genes. In this study, all the reactions were setup using SYBR Green that stain joint to double strand DNA (10 ng concentration) and fluorescent wave was applied until the absorption with acceptor molecule was achieved. The final resulting volume was 20µl containing 4 µl DNA, 2 µl primers, and 14 µl H_2O. The PCR cycling was performed in a gradient the rmocycler with an initial denaturation step at 94 ºC for 2 min followed by 35 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). Continuous monitoring of fluorescent signal was carried out at the last step. During this period, a rapid decrease in the fluorescence occurred due to the denaturation of the amp licons, such that a single strand of DNA appeared with the successive detachment of the SYBR-Green, as was previously described (Matsonaga et al., 1999).

In order to compare the result of the real-time PCR method for quantitative detection in the meat products, a standard curve was drawn based on mixing different percentages of chicken cream (5%, 10%, 50%, 90%) to the cattle and sheep meat which were used in the forms of raw and heated (70 °C for 60 min) (Rodriguez et al., 2005; Rojas et al., 2009). Finally, 24 samples including 18 beef sausage and 6 beef burgers were subjected to the Q-PCR.

In order to determine the detection limit, different concentrations of chicken cDNA were prepared and

subjected to RT-PCR. β-actin gene was employed as an internal control.

RESULTS AND DISCUSSION

Specificity and Sensitivity of The Primers

The primers were designed from specific fragments (450 bp) of chicken DNA and their specificity was confirmed by gene sequencing. Fig. 1 shows that PCR amplification from 0.01, 0.1, 1 ng DNA was clearly amplified the species-specific amp licons up to 0.01 ng concentration of the extracted DNA.

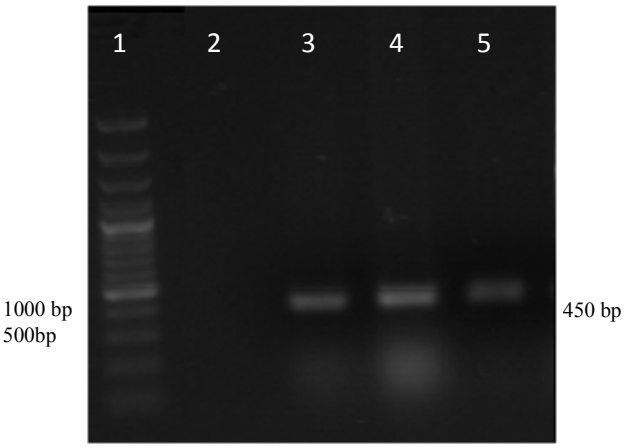

Fig. 1. Detection of 450 base pair *12S rRNA* in meat preparations: the comparison between different proportions of the specimens at concentrations of 1 ng, 0.1 ng and 0.01 ng using conventional PCR (Lane 1: 100 base pair Marker, Lane 2 negative control (no template), Lane 3, 1 ng, Lane 4, 0.1 ng and Lane 5, 0.01ng concentration of DNA.

Detection of Chicken Meat in the Samples

A total of 150 meat products containing different percentages of red meat were monitored using PCR, of which, 84% and 29 % of the sausages and burgers respectively contained chicken meat (Table 1).

Table 1. Detection rates of mislabeled samples (containing chicken meat) of meat products using PCR

Type of meat product	Red meat percentage (according to the lables)	Number of samples	Number of mislabled samples (%)
Sausages	40	16	16 (100)
	55	24	22 (91.7)
	60	20	16 (80.0)
	70	18	16 (83.3)
	80	10	4 (40.0)
	90	12	10 (83.3)
Burgers	30	20	5 (25.0)
	60	15	6 (40.0)
	90	9	2 (22.2)

Real Time PCR System Set Up

The mitochondrial gene encoding of the 12*S rRNA* was chosen as a target for chicken DNA quantification (Grish et al., 2005). As shown in Fig. 2, different concentrations of raw and heated chicken cream mixtures with red meat were detected based on the threshold cycle (Ct) of each sample which is the cycle number where the samples fluorescent curve jumps sharply upward and corresponds to the initial concentration of DNA. In general, the higher the Ct is, the lower the initial concentration of DNA will be. The technique was robust enough to detect 0 to 90 % ratios of the chicken tissues in experimentally made sausages (Fig. 2).

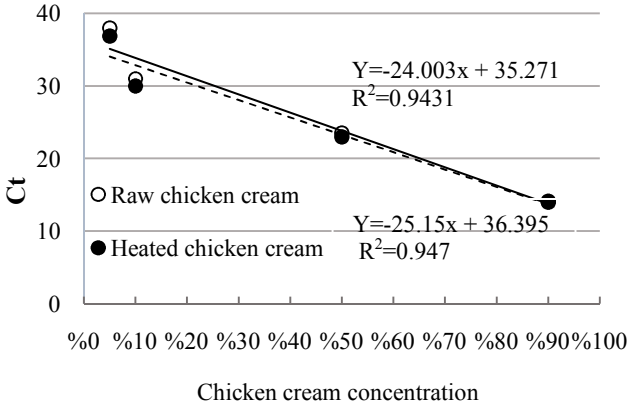

Fig. 2. Standard curve of quantification profiles of different concentration of raw and heated chicken cream (70 °C for 60 min) in experimentally prepared food stuffs

Application of the Technique to Commercial Meat Products

The standard curve of threshold cycle (Ct) approach generated from 5%, 10%, 50% and 90% concentration of chicken meat was used for determining unknown amounts of chicken target DNA in foodstuffs. This curve illustrates the concentrations of standard samples with DNA concentration (10 ng) against their crossing point. Then, quantification measurements were developed with comparable amounts of this Ct values with Ct values of sausages and burgers. The percentage of chicken meat in the specimens collected from retail markets were 10 or 50% (Table 2).

Table 2. Details of the measurements of chicken meat in the meat products using Q-PCR

Type of meat product	% of Red meat*	Threshold cycle (Ct)				Estimate percent of chicken meat in the mislabeled samples
		Mean	S.D.	Min.	Max.	
Sausages	40	30.33	1.25	29.44	31.21	10
	55	23.75	1.93	22.15	25.89	50
	60	21.59	4.88	16.17	25.64	50
	70	28.71	1.34	27.87	30.25	10
	80	24.41	1.90	23.21	26.6	50
	90	29.04	0.57	28.64	29.44	10
Burgers	30	30.79	4.12	27.87	33.7	10
	60	22.50	3.00	19.09	24.76	50

*According to the labels

CONCLUSIONS

Nowadays, the detection of cheating is very important because consumers 'knowledge of food has increased. They like using safe, nutritional and ready-to-use food. Molecular methods are quick, specific and reliable for detection of fraud especially in cooked meat. PCR as a method for *in vitro* amplification of DNA has successfully been used for species identification of plant, bacteria and animals (Aguado et al., 2001; Verkaar et al., 2002). PCR technique extensively distinguished species-specific meat of different animal slike cow, pig, bird, sheep, goat and horses. Calvo et al. (2001) used PCR assay to detect pig and cow meat in raw blend meat, sausage, burgers and canned food. The proposed method allowed the quantification of pork meat in addition to cow meat with a sensitivity of 0.1%.

In this study, we used PCR for fast food composition and authenticity assessment that is a very important issue to avoid unfair competition among producers.

An analysis of experimental chicken meat mixtures demonstrated the suitability of the assay for the detection of the target DNA in the range 1- 0.01%. If chicken cream is used in red meat sausages and burgers, it allows consumers to have incorrect information about the acquired products. According to this research, meat species adulteration in commercial products has been a widespread problem and showed monitoring and management programs are not efficient in Iran.

Due to the fact that the conventional PCR assay is a qualitative technique, here, we have employed a real time PCR assay to quantify the amount of chicken meat in the final products (Kesman et al., 2007).

Brodmann and Moor (2003) explained that RT PCR allows the detection and quantification of the smallest amounts of beef DNA in most food and food products (sausage, canned meat, modified bone meal). TaqMan real-time polymerase chain reaction systems used for the detection and quantification of bovine, porcine, lamb, chicken, turkey, and ostrich DNA in complex samples was also employed (Lopez-Andreo et al., 2005). Fajardo et al. (2008) have developed a SYBR Green Real time PCR for the quantification of red deer, fallow deer, and roe deer DNAs in meat mixtures in the range of 0.1–0.8% .

Martin et al. (2009), Sawyer et al. (2003) and Fajardo et al. (2008) clearly demonstrated the suitability of the specific- primer SYBR Green assay for the detection and quantification of species DNA in raw and processed mixtures containing as little as 0.1% and this method is a practical, accurate, simple, and relatively quick method with good sensitivity and specificity in such conditions.

The presence of targeted DNA was successfully identified in each sample, and the amplification was not affected by the use of spice and other ingredients in the mixtures. Similar results were also found in the fermented sausages using the same primers, elsewhere (Kesman et al., 2006).

The amount of chicken meat in products is very important because following labelling regulations, fraudulent substitution of higher commercial value meats by lower value meats is cheating. In this work, the rate of chicken meat in samples was 50% maximally that must be taken into consideration.

In order to detect different proportions of chicken cream, a real time PCR was also employed. Using a quantification method based on a mitochondrial sequence, a species-specific target genome as an indicator of the total DNA was also explained (Lopez-Andreo et al., 2005). SYBR Green in real time PCR is a dye that binds to the minor groove of double – stranded DNA. When bound to double – strand DNA, SYBR Green is highly fluorescent (Ballin et al., 2009).

These results clearly revealed that specific SYBR Green real time PCR method is a sensitive, specific, convenient and cheap assay for rapid quantification of chicken cream in meat adulteration.

In conclusion, the SYBR-Green real-time PCR system provided an applicable technique to detect different concentrations of chicken materials in food products and thus could be proposed as a reliable technique to prevent frauds in the meat industries. The technique is strongly recommended for the routine analysis of cooked meat products as well.

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شناسایی و اندازه‌گیری مقادیر غیر مجاز خمیر مرغ در محصولات گوشتی شامل سوسیس و برگر با استفاده از روش سایبرگرین -RT PCR(Q-PCR)

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واژه‌های کلیدی:
مرغ
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چکیده– امروزه توجه به تولید محصولات گوشتی بدون تقلب و با هزینه‌ی کمتر افزایش یافته است. بنابراین با در نظر گرفتن نگرانی‌های مربوط به سلامت عمومی اعتقادات مذهبی و مسائل اقتصادی، لازم است اقدامات مناسب جهت ممانعت از این تقلبات به عمل آید. در مطالعه‌ی حاضر با استفاده از آزمایش Real Time PCR به عنوان یک روش سریع، حساس و اختصاصی، به شناسایی و اندازه‌گیری مقدار استفاده از بافت‌های مرغی در محصولات گوشتی با برچسب تهیه شده از گوشت قرمز پرداخته شده است. بدین منظور، بر اساس ژن *12s r*RNA ، پرایمرهای اختصاصی آماده گردید و با روش‌های PCR و RT-PCR بر روی تعداد ۱۵۰ DNA استخراج شده از نمونه‌های سوسیس و برگر انجام گردید. نتایج حاصل از مطالعه‌ی حاضر نشان دهنده‌ی وجود ۸۴٪ و ۲۶٪ بافت مرغ به ترتیب در نمونه‌های سوسیس و برگر می‌باشد. به علاوه روش RT-PCR(Q-PCR) قادر به تشخیص تقریبی ۵-۱۰٪ بافت مرغ در این محصولات است. این محدوده‌ی تشخیص توسط روش فوق می‌تواند به طور مؤثری توسط مراجع ذی‌صلاح به منظور کنترل کیفیت محصولات گوشتی مورد استفاده قرار گیرد.