A sensitive multiplex PCR protocol for simultaneous detection of chicken, duck, and pork in beef samples

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Abstract A rapid and sensitive multiplex PCR assay was developed for simultaneous identification of the adulteration ingredients of chicken, duck and pork in beef. Specific primers for the mitochondrial genes of Cyt b, CO III, ATPase subunit 8/6 and Cyt b of chicken, duck, pork, and beef, respectively, were adopted in the assay. DNA extraction from meat samples was carried out by using magnetic nanoparticles as rapid separation substrates. The multiplex PCR assay showed that the limit of detection was 0.05% for each species. Moreover, the multiplex PCR specifically identified five beef samples adulterated with pork and one beef samples adulterated with chicken among the 35 commercial samples examined, indicating the practicability of this multiplex PCR method for identifying adulterated ingredients of chicken, duck, and pork in commercial beef products.

Keywords Multiplex PCR · Identification · Chicken · Duck · Pork · Beef · Meat adulteration · Sensitivity · Specificity

Introduction

Food composition authentication is an important part of food safety and quality assessment (Safdar et al. 2014). With increasing meat products in people's diet, the authenticity of meat products has become a major issue for consumers. Expensive meats may be adulterated with cheap or poor-quality meats such as chicken, duck, and pork for illegal economic profit (Hitchcock and Crimes 1985; Fajardo et al. 2010). Meat adulteration not only causes economic losses, but also may affect consumers' health. For example, consumers are afraid for adulterated meat products because of the spread of diseases such as avian influenza virus (Das et al. 2008), swine influenza (Myers et al. 2007), and foot and mouth disease (FMD) (Zhang et al. 2010). Also, for consumers who are allergic to specific meats may be caused severe allergic reactions by the uncertain meat compositions (Choi et al. 2007; González-Mancebo et al. 2011; Chinuki et al. 2015). Besides the economical and health aspects, meat adulteration is a taboo for consumers who have dietary restrictions due to religious practices or ethnical options (Teletchea et al. 2005). For example, there are 1.5 billion Muslims around the world and they are only allowed to consume Halal food in which pork is strictly forbidden even in minute quantities (Nakyinsige et al. 2012). Take these into consideration, identification of ingredients of chicken, duck and pork in beef products with rapid, sensitive, and accurate methods is of great importance.

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So far, numerous DNA based analytical methods have been developed for identification of meat adulteration because of its high thermal stability (Blake and Delcourt 1998). Recently, polymerase chain reaction (PCR) based DNA protocols for food analysis have been increased, presenting a fast, sensitive and specific alternative and enabling the identification of species in complex processed foods (Fajardo et al. 2010). As a result, different methods such as PCR (Karabasanavar et al. 2013), PCR restriction fragment length polymorphism (PCR-RFLP) (Girish et al. 2005; Verkaar et al. 2002), random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Martinez and Yman 1998), and PCR-southern hybridization (Mutalib et al. 2015) have been applied for identification of meat adulteration. However, these methods have inferior reproducibility, complex operation or low efficiency for simultaneous multiple detection of various meat species. Nowadays, real-time PCR is probably the most common method for quantification of trace target due to its fast, highly sensitive and specific properties (Xu et al. 2018; Rojas et al. 2011). However, its application is limited by the relative high cost of reagents and equipment than the conventional PCR. Meanwhile, the detection throughput should be seriously considered in view of the ever-increased detection requirements for rapid, economical, and simple food safety monitoring. Therefore, it is necessary to establish robust multiplex conventional PCR (multiplex PCR) methods for easy authentication of meat adulterations, which can realize simultaneous detection of several targets with one reaction system (Henegariu et al. 1997; Matsunaga et al. 1999; Sun et al. 2007) and overcome the weakness of simplex PCR (one target in one reaction). Compared with real-time PCR, multiplex PCR requires only regular PCR primers without any modification, which saves the time and reduces the cost of detection (Kitpipit et al. 2014; Hou et al. 2015; Safdar and Junejo 2016; Izadpanah et al. 2018).

In this study, to develop a multiplex PCR method for simultaneous identification of adulterated ingredients of chicken, duck, and pork in beef is reported for food quality guarantee. By using specific primer set for mitochondrial genes of chicken, duck, pork and beef respectively, the results have shown that this multiplex PCR provided a very effective tool for avoiding the adulteration fraud, and held a great potential for rapid, cost-effective, sensitive, and specific identification of multiple meat components and meat derived products.

Materials and methods

Preparation of samples

All samples used in this work were obtained from commercial sources. Initially, raw meat samples, including chicken, duck, pork, beef, mutton, horse, turkey, goose, dog, donkey, rabbit and pigeon were cut into small pieces and dried at 70 °C. Then, the dried meat samples were milled into powder and stored at – 20 °C. To validate the multiplex PCR, the adulterated meats were prepared by blending powdered chicken, duck, and pork into beef together with a series of proportion: 30%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, and 0% (Of note, this is for the single adulteration sample preparation. For multiple adulteration sample preparation, taking 30% adulteration of each component as example, the final proportion of beef is only 10% when three adulterated components exist simultaneously). To investigate the feasibility of multiplex PCR, the processed beef samples was prepared by mixing wet raw chicken, duck or pork in beef, and autoclaved at 103.4 kPa and 121 °C for 30 min (He et al. 2015).

Magnetic nanoparticles (MNPs) based DNA extraction

The meat DNA templates were extracted and purified by using carboxylated MNPs as the separation substrates, which were prepared in our laboratory according to a previously report (Zhao et al. 2012; Huang et al. 2015). Briefly, 50 mg of meat samples were mixed with 700 µL of extraction buffer (10 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% SDS, pH 8.0) and 30 µL Proteinase K (20 mg/mL). After incubation at 65 °C for 1 h, the mixture was centrifuged at 12,000 g at 4 °C for 10 min, and the supernatant were transferred to a new 1.5 mL sterile tube which contained 300 µL of PEG/NaCl solution. Then, 20 µg carboxylated MNPs were added to the solution to separate and purify the DNA. After 5 min, the MNPs were collected under magnetic field and washed twice with ethanol, and then dispersed in 100 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) for subsequent use. The extracted DNA was analyzed by UV/vis spectrophotometry taking O.D. 260–280. Notably, the soybean and rice were initially crushed and then same extracted as the animal origin ones.

Primers

Specific primers for mitochondrial gene of chicken, duck, pork and beef were used based on previously published reports (Zhang et al. 2008; Li et al. 2004; Fan et al. 2013).

All primers were purchased from Sangon Biotech (Shanghai, China, www.sangon.com). The specificity of each primer was verified by BLAST program.

PCR amplification and gel electrophoresis analysis

PCR was carried out using a S1000 Thermal Cycler PCR (Bio-Rad, Hercules, CA, USA). The single PCR was performed in a total volume of 25 μ L, containing 1 $\times$ PCR buffer, 2.5 mM $MgCl_2$, 200 μ M of each dNTP, 0.08 μ M of each specific primer, 1 U Taq DNA polymerase and 50 ng of DNA template. Multiplex PCR was performed in a total volume of 25 μ L, containing 1 $\times$ PCR buffer, 2.75 mM $MgCl_2$, 200 μ M of each dNTP, optimized concentrations of four primer pairs, 1.5 U Taq DNA polymerase, and 200 ng of DNA template. PCR was performed under the following conditions: denaturation at 94 $^{\circ}C$ for 5 min, followed by 30 cycles of 94 $^{\circ}C$ for 30 s, 60 $^{\circ}C$ for 30 s, and 72 $^{\circ}C$ for 30 s. A final extension was performed at 72 $^{\circ}C$ for 5 min. In order to validate the amplification and authentication, the band of beef component at 91 bp is adopted as the positive controls, indicating the successful amplification for authentication and the existence of beef component. For real applications, the positive control can be chosen based on the specific adulterated meat sample for the validation of authentication. In this case, if no amplification occurred, this specific meat sample is totally a fake sample rather than an adulterated one or the application is not well performed for authentication.

Following amplification, PCR products were analyzed with 2% agarose gel electrophoresis. Briefly, 10 μ L loading samples consisting of 8 μ L tested amplification product and 2 μ L 5 $\times$ loading buffer were subjected to the gel lanes, and the electrophoresis was conducted in 1 $\times$ TBE buffer at a constant voltage of 150 V for 30 min. The final gel was imaged and analyzed by LG 2080 Gel imaging analysis system.

Optimization of the multiplex PCR parameters

Unless otherwise stated, the optimal conditions of multiplex PCR including the annealing temperature, Mg^{2+} concentration, and primers pair concentration were all pre-assessed. For instance, under fixed concentrations of Mg^{2+} and primers, the optimization of annealing temperature for multiplex PCR was performed via setting a series of different annealing temperature (63.0 $^{\circ}C$, 62.5 $^{\circ}C$, 61.5 $^{\circ}C$, 60.0 $^{\circ}C$, 58.2 $^{\circ}C$, 56.9 $^{\circ}C$, 55.8 $^{\circ}C$, and 55.0 $^{\circ}C$). Other procedures were identically performed as that described in “PCR amplification and gel electrophoresis analysis” section. Similarly, the Mg^{2+} concentration was optimized under the fixed annealing temperature and primers pair concentration, while the primers pair concentration was

optimized at a fixed annealing temperature and Mg^{2+} concentration. Notably, the band brightness of the PCR products of four different DNA templates is the sole criterion to judge the optimal experimental conditions.

Specificity and sensitivity evaluation

To verify the specificity of primer sets, the PCR amplification process was conducted as that of single PCR in “PCR amplification and gel electrophoresis analysis” section but under optimal PCR conditions. And for each of the specific primer set, it should be tested for 14 samples with the presence of extracted chicken, duck, pork, beef, mutton, horse, turkey, goose, dog, donkey, rabbit, pigeon soybean, and rice DNA templates, respectively. The negative amplification was performed by using ddH₂O instead of the extracted DNA. The resultant amplicons were directly characterized by 2% agarose gel analysis. Next, the DNA templates extracted from these mixtures were used for the multiplex PCR amplifications.

Spiked and commercial samples identification

A series of blind spiked samples were prepared by blending the fresh meats of chicken, duck, and pork with beef. Afterwards, the mixture was autoclaved and treated for DNA extraction. For commercial purchased beef products, they were directly employed for DNA extraction without any pretreatments. The obtained DNA templates were utilized for multiplex PCR amplification.

Results and discussion

Optimization of multiplex PCR

To achieve an optimal analytical performance, several parameters of multiplex PCR including annealing temperature, Mg^{2+} concentration, and primer concentration were investigated. As shown in Fig. 1a, with the annealing temperature changed from 63.0 to 55.0 $^{\circ}C$, the bands of chicken, duck, and beef all weakened gradually while the band of pork kept the brightness steadily. Considering the possible non-specific recognitions and poor hybridization efficiency between primers and DNA templates aroused by low and high annealing temperature, respectively, 60 $^{\circ}C$ was selected as the optimal annealing temperature for the multiplex PCR. For Mg^{2+} concentration optimization (Fig. 1b), the bands of multiplex PCR enhanced clearly with the increasing concentration of Mg^{2+} and came to a saturated value at 2.75 mM. This is reasonable since the increase of Mg^{2+} concentration would improve the activity of polymerase and led to a more efficient PCR

Fig. 1 **a** Optimization of the annealing temperature. M, DNA marker; lanes 1–8, 63.0 °C, 62.5 °C, 61.5 °C, 60.0 °C, 58.2 °C, 56.9 °C, 55.8 °C and 55.0 °C, respectively; lane 9, negative control, amplified with ddH₂O at 60.0 °C. **b** optimization of Mg²⁺ concentration. M, DNA marker; lanes 1–7, 1.5 mM, 1.75 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM and 3.0 mM; lane 8, negative control, amplified with ddH₂O, 2.5 mM; **c** optimization of chicken, beef, pork, and duck primer pairs concentration, respectively. M, DNA molecular marker; lanes 1–7, 0.02 μM, 0.04 μM, 0.08 μM, 0.12 μM, 0.20 μM, 0.28 μM and 0.40 μM, respectively; lane 8, negative control amplified with ddH₂O, 0.08 μM

amplification. However, excessive use of Mg²⁺ was not meaningful. Thus, 2.75 mM Mg²⁺ was adopted as the optimal concentration. The results of concentration optimization of chicken, beef, pork, and duck primer pair were shown in Fig. 1c. From Fig. 1c, one can find that with the increase concentration of chicken primer pair, the band brightness increased gradually and remained a saturated intensity since 0.12 μM, while the amplification for other species were significantly inhibited because of the superior competitive effect of chicken primer. Similarly, it was easy to find that the optimal concentrations of beef, pork, and

duck primer pairs were 0.20 μM, 0.08 μM, and 0.20 μM, respectively. In these conditions, a good competition balance between the four primers pairs is constructed for achieving easily distinguished bands.

Specificity study of the multiplex PCR

In order to verify the specificity of primer sets, each pair of primers was amplified with the different extracted DNA of chicken, duck, pork, beef, mutton, horse, turkey, goose, dog, donkey, rabbit, pigeon, soybean, and rice DNA

templates, respectively. Originally, the specificity of each primer set to each adulterated component is only confirmed by the corresponding components. Herein, the adopted four primer sets are further interrogated with other 10 common components in our daily foods. The image results of agarose gel electrophoresis analysis for 14 DNA templates were shown in Figure S1 and a mass of dispersed DNA bands in each lane confirmed the successful extraction of DNA templates. Meanwhile, as the amplification results in Fig. 2, with the four pairs of specific primers, only corresponding investigated species DNA of each component (duck, chicken, pork and beef) in this study can be amplified with occurrence of target band whereas the others were not amplified, indicating that the four primer sets all have excellent specificity for simultaneous authentications. Then, for simultaneous detection, the specificity of multiplex PCR was further evaluated based on the results in Fig. 2. As shown in Fig. 3, it can come to the following conclusions (1) all target genes of different component can be simultaneous amplified together; (2) for single, double and multiplex detection, different target genes can be well amplified without any cross-reactivity. The desirable results are ascribed to the fact that the stringent specific hybridizations make no-mismatched hybridizations between the primer pair and its non-target DNA template

exist, demonstrating that this optimized multiplex PCR is reliable and specific for practical applications.

Sensitivity of the multiplex PCR for adulteration authentication

Under the optimal conditions, the sensitivity of multiplex PCR was investigated by blending powdered chicken, duck, and pork into beef with a series of proportions: 30%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0%. Positive control was performed with pure chicken, duck, and pork DNA, respectively. Negative control was amplified with ddH₂O. Given the decreased multiplex PCR amplification events (Fig. 4), the bands of chicken, duck, and pork were weakened gradually with the decrease of adulteration proportion. The clear bands can be distinguished at 0.05%. Notably, the obtained detection limit is powerful enough to meet the public demand and restrict the commercial wrongdoings since the adulteration percentage of 0.05% can only create negligible earnings. One thing should be noted is that this excellent LOD is obtained with the dried and milled samples. If evaluated based on the weight of the wet minced or fresh hydrated meat samples, the final LOD may be a little different from that of the dried samples. The high sensitivity of this method can be attributed to the utilization of MNPs for DNA purification from meat

Fig. 2 The specificity of the primers pair used in the PCR assay. **a–d** Agarose gel electrophoresis of single PCR product amplified with chicken, duck, pork and beef primers pairs, respectively. M, DNA molecular marker; lanes 1–8, DNA templates from chicken, duck,

pork, beef, mutton, horse, soybean and rice, respectively; lane 9, negative control, amplified with ddH₂O; lanes 10–15, DNA templates from turkey, goose, dog, donkey, rabbit and pigeon, respectively

Fig. 3 The specificity of the multiplex PCR assay. Agarose gel electrophoresis of multiplex PCR product amplified with different templates. M, DNA marker; lanes 1–12, DNA template from chicken, duck, pork, beef, mutton, horse, soybean, rice, chicken + beef,

duck + beef, pork + beef, and chicken + duck + pork + beef, respectively; lane 13, negative control, amplified with ddH₂O; lanes 14–19, DNA templates from turkey, goose, dog, donkey, rabbit and pigeon, respectively

Fig. 4 The sensitivity of the multiple PCR assay. M, DNA marker; lane 1, chicken; lane 2, duck; lane 3, pork; lanes 4–12, the adulteration proportion of chicken, duck, and pork in beef are 30%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0%, respectively; lane 13, negative control, amplified with ddH₂O

samples, which does not contain any inhibitor of PCR. Nevertheless, the traditional extraction method may contain residual solvents, such as chloroform and phenol, leading to the inhibition of multiplex PCR amplification at a certain degree. Detailed comparisons of the current multiplex PCR with reported methods for meat adulteration analysis were provided in Table S1. Most of the reported methods can only realize detection of only one component at each detection while other can achieve detection of two or three analytes simultaneously with the sacrifice of sensitivity. One can easily find that minimum detection limits of 0.1% or 1% for adulterated meats detection were found in different literatures but with fewer detection species or costly instruments, suggesting the proposed method possesses improved assay performance in simultaneous

authentication of adulterated species in beef without any expensive or professional instruments.

Adulteration determination of the processed beef products

In order to verify the practical applicability of this multiplex protocol for adulteration, some spiked blind samples by mix one, two, three, or four different kinds of meats and some other common meat components were prepared, recorded, and autoclaved by others rather than the analyst (seen in “Preparation of samples” section). Then, the spiked samples were determined with this multiplex protocol. Detection results of the processed beef samples with this developed method has been demonstrated in Fig. 5a,

Fig. 5 a Testing of processed beef products by the multiplex PCR assay. M, DNA molecular marker; lane P, positive control; lanes 2–21, processed beef samples; lane N, negative control, amplified with ddH₂O. **b** Testing of commercial beef products by the multiplex PCR assay. M, DNA molecular marker; lane P, positive control; lane N, negative control, amplified with ddH₂O; lane 1, beef tendon; lanes 2–4, beef strip; lanes 5–6, beef jerky; lane 7, beef granules; lanes 8–9, chilled beef; lane 10, beef ball; lane 11–13, beef granules; lane 14–20, beef ball; lane 21–24, beef slices; 25–27, beef jerky; lane 28–30, beef strip; lane 31–35, beef barbecue

showing that 3/21 samples are contaminated with duck contents, 7/21 samples are contaminated with pork contents, 3/21 samples are contaminated with chicken contents

and 2/21 samples (line 9 and 12) are adulterated with other meat components except the four components in this

research. As expected, all these results are consistent with the initial spike conditions with different meat components.

To further confirm the practicability of the assay, 35 commercial beef products that labeled as 100% pure beef were also tested, the details of all commercial beef samples are listed in Table S2. As showed in Fig. 5b, five commercial sample were identified with pork and one commercial sample was identified with chicken among 35 samples. The strong bands of pork and chicken were most likely the intentional practice at manufacturers level. These samples are also confirmed with RT-PCR (Safdar and Abasiyanik 2013) and same conclusions are achieved, indicating that our multiplex PCR method could be used for the real-word identification of commercial beef products (raw or ripen) for quality assurance and certification.

Conclusion

In summary, we have developed a systematic optimized multiplex PCR protocol for simultaneous detection of adulterated components including chicken, duck and pork in beef samples. Compared to earlier reported approaches, our method demonstrates several distinctive advantages. The multiplex PCR method can be used to detect chicken, duck and pork from beef simultaneously in one single reaction without any additional processing aids, such as, special expertise, unique equipment or reagents. And the detection results of the assay can be simply characterized by gel electrophoresis, which greatly minimizes the cost for analysis. The LOD of the multiplex PCR is as low as 0.05%, which indicates this method is highly sensitive and reliable (Details in Table S1). However, for the authentication of the unknown components, a wide range of primer set bank is required for the simultaneous analysis. Besides, the throughput of the multiplex PCR protocol cannot be increased infinitely, which can increase the risk of cross reactivity and non-specific amplifications. We believe that this multiplex PCR assay may be used by food regulatory agencies and analytical laboratories, especially for the resource-limited laboratories, for tracing ingredient of chicken, duck, pork in beef and beef products.

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