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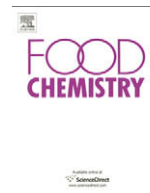

Analytical Methods

Polymerase chain reaction assay for identification of chicken in meat and meat products

B.G. Mane [a,*], S.K. Mendiratta [a], A.K. Tiwari [b]

[a] Division of Livestock Products Technology, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP 243 122, India
[b] Division of Animal Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP 243 122, India

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ABSTRACT

The aim of this study was to develop polymerase chain reaction (PCR) assay for specific detection of chicken meat using designed primer pair based on mitochondrial D-loop gene for amplification of 442 bp DNA fragments from fresh, processed and autoclaved meat and meat products. The PCR result was further verified by restriction digestion with *Hae*III and *Sau3A*I enzymes for specific cutting site in amplified DNA fragments. The specificity of assay was cross tested with DNA of cattle, buffalo, sheep, goat, pig, duck, guinea fowl, turkey and quail, where amplification was observed only in chicken without cross reactivity with red meat species. However positive reaction was also observed in quail and turkey. In this study, no adverse effects of cooking and autoclaving were found on amplification of chicken DNA fragments. Thus, the detection limits was found to be less than 1% in admixed meat and meat products. The developed assay was found specific and sensitive for rapid identification of admixed chicken meat and meat products processed under different manufacturing conditions.

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1. Introduction

The adulteration/substitution of meat has always been a concern for various reasons such as public health, religious factors, wholesomeness and unhealthy competition in meat market (Arslan, Irfan-Ilhak, & Calicioglu, 2006; Mane, Tanwar, Girish, & Dixit, 2006). Consumer should be protected from these malicious practices of meat adulterations by quick, precise and specific identification of species present in meat and meat products. Various methods are employed for detection of species origin of meat. These include sensory evaluation to latest DNA based assays. DNA based assays are gaining popularity in meat species identification due to their stability at high temperature and conserved structure within all individual of the species (Calvo, Zaragoza, & Osta, 2001; Girish et al., 2004). Earlier, DNA based assay employed for identification of species origin of meat was DNA hybridisation (Baur, Teifel-Greiding, & Leibhardt, 1987) but nowadays that has been replaced by PCR assays.

The PCR assays are employed for identification of species origin of meat using random primers (Saez, Sanz, & Toldra, 2004) to amplify the non-targeted DNA, while universal primers are used (Girish et al., 2005; Verkaar, Nijman, Boutaga, & Lenstra, 2002) for amplification of targeted genomic and mitochondrial DNA followed by restrictive enzyme digestion to differentiate meat species. The results of RAPD–PCR are non-reproducible due to requirement of high stringent conditions (Koh, Lim, Chua, Chew, & Phang, 1998), while PCR–RFLP is more time consuming, require more analytical work and result interpretation is complex. The result interpretation is even more complex in admixture meat and meat products (Irfan-Ilhak & Arslan, 2007). The specific detection of species origin of meat by PCR using species-specific primers is relatively quick, precise, sensitive and cost effective as compared to other PCR based assay (Mane et al., 2007). This species-specific PCR assay was previously used by various workers for detection of various mammalian and poultry species in meat and meat products (Arslan et al., 2006; Meyer, Candrian, & Luethy, 1994).

These PCR assays targets genomic as well as mitochondrial DNA for the purpose of meat species identification, even in cooked meat under different processing conditions. However, in the present study the mitochondrial DNA was used for meat species identification because of the maternal inheritance of mitochondria, normally only one allele exists in an individual and thus no sequence ambiguities are expected from the presence of more than one allele (Unseld, Beyermann, Brandt, & Hiesel, 1995). The variable regions of the mitochondrial gene are present in thousands of copies per cell (Greenwood & Paboo, 1999), which increases the probability of achieving a positive result even in severe DNA fragmentation due to intense processing conditions (Bellagamba, Moretti, Comincini, & Valfre, 2001). Keeping the above facts and considering future implications in the meat trade, the present study was planned with the overall objective to develop simple, quick, sensitive, precise, cost effective and applicable method for identification of chicken meat and meat products processed under different manufacturing conditions by PCR assay.

* Corresponding author. Fax: +91 581 2447284.
E-mail address: drbandu_176@rediffmail.com (B.G. Mane).

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2. Materials and methods

2.1. Meat and blood samples

The fresh meat samples of buffalo, sheep, goat, pig, chicken and duck were collected from the local municipal slaughterhouses. While the guinea fowl, turkey and quail samples were collected from central avian research institute. After collection, samples were kept at −20 °C till further processing. The blood samples were collected from specific breeds of species under investigation from different sources. The blood was collected in sterile 15 ml polypropylene tube containing 0.5 ml of 0.5 M ethylene diamine tetra acetate (EDTA) solution, which acts as an anticoagulant. The collected blood samples were preserved at −20 °C till DNA isolations. The DNA samples of certain breeds were also collected from different labs of this institute.

2.2. DNA extraction

DNeasy® Blood and Tissue Kit (Qiagen, USA) was used for extraction of genomic DNA from meat and blood as per the instructions given by manufacturer. The same kit was also used for extraction of DNA from processed and cooked meat and meat products.

2.3. Checking quality and purity of DNA

The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis using 0.8% agarose. The purity of genomic DNA was checked by using spectrophotometer taking $O.D._{260-280}$.

2.4. Oligonucleotide primer pair

In the present study, self designed primer pair based on the mitochondrial D-loop gene sequences of chicken was used. The published DNA sequences of the different species were retrieved from the National Center for Biotechnology Information (NCBI) GenBank for designing of primers. The chicken-specific primer pair was designed using primer designing soft-ware (DNA-STAR Inc., USA). The primer pairs designed were synthesised from Metabion International, Germany. The details of primer pair used in the present investigation are given below:

- **Forward:** 5′ CTC CCC ATA GAC AGC TCC AAA C 3′
- **Reverse:** 5′ CCC CAA AAA GAG AAG GAA CCA A 3′

2.5. Polymerase chain reaction (PCR)

The reaction mixture was prepared in a 500 μl PCR tube (AXYGEN, USA) in a total volume of 50 μl containing 5 μl of 10X PCR buffer, 200 μM each of dNTP, 1–2 Units of *Taq* DNA polymerase (Qiagen, USA), 10–20 pmol each of forward and reverse primer, 1 μl of DNA template (20–30 ng) and remaining nuclease free water (Fermentas, USA).

The PCR conditions programmed on master cycler gradient thermocycler (Eppendorf, Germany) were as follows: initial denaturation at 94 °C for 2 min followed by 30–35 cycles of denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min and extension at 72 °C for 1 min. Then final extension was done at 72 °C for 5 min. The PCR product was kept at −20 °C for further use.

2.6. Electrophoresis of PCR products

The submarine horizontal agarose gel electrophoresis was used for analysis of PCR products. Two percent agarose was used for preparation of gel. For that 0.4 g of agarose (Ambion, USA) was put in 20 ml of 1× TBE solution (Fermentas, USA.) and heated to completely dissolve the agarose. Then 1 μl (5%) ethidium bromide solution was added as gel visualising agent and mixed thoroughly. The electrophoresis was done for 90 min at 80 V. The PCR product was finally analysed using UV transilluminator and documented by gel documentation system (Alpha Imager, USA). The ready to use 100 bp ladders (Fermentas, USA) was used for present work.

2.7. Characterisation of PCR fragments

The results of PCR assay was further confirmed by digestion of PCR products with *Hae*III and *Sau*3AI restriction enzymes based on available gene sequences of D-loop regions in NCBI database. For RE digestion first, the PCR products were purified by PCR purification kit and then restriction digestion reaction was assembled by adding the given reagents in following order: Nuclease free water, PCR product (10–20 μl), 10× enzyme buffer (EB) and restriction enzyme. The reaction mix was incubated overnight at 37 °C in water bath. The digestion reaction was stopped by adding 6× loading dye. The products were kept at −20 °C till electrophoresis. The digested products were subjected to electrophoresis in 2.5% agarose gel along with 100 bp ladder (Fermentas, USA). Finally, after electrophoresis, the gel was observed for desired band pattern and documented by gel documentation system.

2.8. Sensitivity and specificity of PCR assay

The specificity of PCR assay was tested with DNA of other meat species used in this study, while the sensitivity of the assay was tested in admixed meat and meat products containing 10%, 5%, 1% and 0.1% chicken in meat and meat products. The non-targeted species in the admixed meat and meat products were beef, buffalo meat, pork, chevon and mutton.

2.9. Preparation of emulsion based meat products

Meat emulsion of about 2.5 kg was prepared with the help of meat mincer and bowl chopper (Seydelmann K20 Ras, Germany). The pre-weighed quantity of minced meat was blended with 1.7%, 0.4%, 0.4% of common salt, sugar, sodium tripolyphosphate, and 150 ppm sodium nitrite was added and chopping was done for about 1–2 min. Refined vegetable oil (4%) was slowly incorporated during chopping. Condiments paste, spice mixture and maida were also added at 4%, 1.5% and 3%, respectively, and chopping was continued till desired consistency of emulsion was achieved. The condiments paste was prepared from onion and garlic in a ratio of 4:1, while readymade spice mix of reputed brand was procured from the market.

2.10. Cooking and processing under different conditions

In the present investigation meat and meat products were cooked/processed at different temperatures under different conditions to evaluate applicability of standardised PCR assay for different type of meat and meat products. The details of heat treatments, temperature-time combination used for heat treatments of meat and meat emulsions were dry heat in oven at 180 °C for 30 min for meat kabab and meat patty; steam cooking at 100 °C for 45 min for meat block and autoclaving at 121 °C, 15 psi for 15–20 min. After cooking products were stored at −20 °C for further use.

3. Results and discussion

Precise, authentic and rapid testing of animal origin foods is indispensable to avoid unfair market competition and protection of consumer from fraudulent practices of meat adulteration. So,

the aim of the study was to develop and evaluate the species-specific PCR assay for identification of chicken species in meat and meat products processed under different manufacturing conditions. The species-specific primer pair was designed based on mitochondrial D-loop gene for amplification of about 442 bp DNA fragments from chicken meat DNA. The mitochondrial DNA was targeted to design species-specific primers, because mitochondrial DNA is maternally inherited so normally only one allele exists in an individual and thus no sequence ambiguities are to be expected from the presence of more than one allele (Unseld et al., 1995), the variable regions of the mitochondrial gene are present in thousands of copies per cell (Greenwood & Paboo, 1999), which increases the probability of achieving a positive result even in severe DNA fragmentation due to intense processing conditions (Bellagamba et al., 2001), thus making it ideal for identification species origin of processed meat and meat products. Other workers also suggested that mitochondrial markers are more efficient than nuclear markers for the purpose of identification and authentication meat species (Hopwood, Fairbrother, Lockley, & Bardsley, 1999).

The great care was taken during designing primers at 3′ end sequences to avoid nucleotide matching with other meat species. The primer pair successfully optimised for amplification of 442 bp DNA fragment of mitochondrial D-loop gene of chicken using the designed chicken-specific primer pair after repeated testing (Fig. 1). Subsequently, PCR assay was employed for amplification of DNA of available different breeds of chicken to confirm the consistency of amplification pattern. Earlier, various workers have been also successfully employed species-specific PCR assay for identification of species origin of meat targeting nuclear and mitochondrial DNA (Arslan et al., 2006; Frezza et al., 2008; Guoli, Mingguang, Zhijiang, Hongsheng, & Qiang, 1999). The species-specific assays were found to be highly sensitive for identification of species origin of meat reported by Irfan-Ilhak and Arslan (2007). The authenticity of species-specific PCR assay was confirmed by restriction digestion of PCR amplified DNA fragments with *Hae*III and *Sau*3AI restriction enzymes. The amplified 442 bp DNA fragment of DNA extracted from chicken meat was subsequently verified with restriction enzyme digestion with *Hae*III having site at 246 bp resulting into 246 and 196 bp DNA fragments, while *Sau*3AI having cutting sites at 182, 201 and 391 bp resulting into 190, 182, 51 and 19 bp (approximately visible bands at 180–190 bp) fragments (Fig. 2). The verification was done for further confirmation of PCR results by digestion of PCR products with restriction enzymes. The restriction enzymes selected for characterisation of DNA sequence were on the basis of the sequences available in NCBI database with the help of MapDraw programme of Lasergene software (DNA-STAR). The earlier workers also adopted this approach for further confirmation of PCR products amplified from meat and meat products (Guoli et al., 1999; Montiel-Sosa et al., 2000).

The optimised PCR assay was subsequently cross tested with DNA extracted from cattle, buffalo, sheep, goat, pig, duck, guinea fowl, turkey and quail for checking the specificity of the primer pair with chicken, where amplification of 442 bp DNA fragment was observed only in chicken without any cross reaction with red meat species investigated in this study (Fig. 3). However

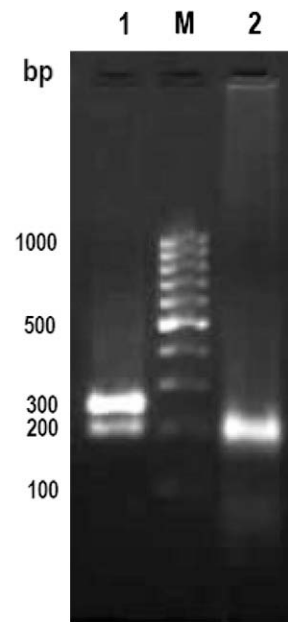

Fig. 2. PCR amplified chicken mitochondrial D-loop gene digested with *Hae*III and *Sau3A*I restriction enzyme. Lane M: 100 bp ladder; Lane 1: *Hae*III; Lane 2: *Sau3A*I.

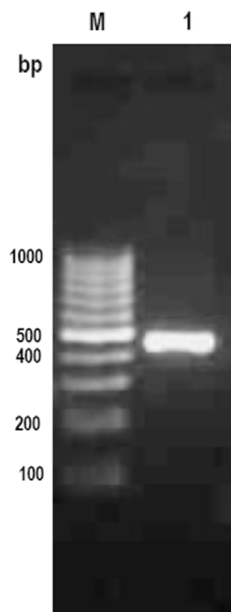

Fig. 1. PCR amplification of chicken mitochondrial D-loop gene extracted from meat. Lane M: 100 bp ladder; Lane 1: chicken.

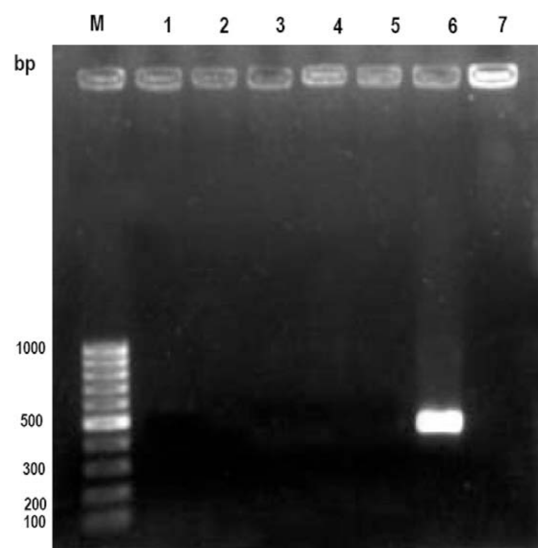

Fig. 3. PCR amplification of mitochondrial D-loop gene with different meat species. Lane M: 100 bp ladder; Lane 1: cattle; Lane 2: buffalo; Lane 3: sheep; Lane 4: goat; Lane 5: pig; Lane 6: chicken; Lane 7: negative control.

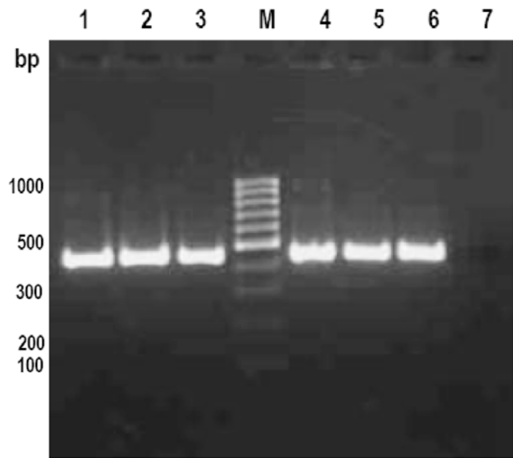

Fig. 4. PCR amplification of chicken mitochondrial D-loop gene with raw, cooked and autoclaved meat and meat emulsion. Lane M: 100 bp ladder; Lane 1: raw meat; Lane 2: cooked meat; Lane 3: autoclaved meat; Lane 4: raw meat emulsion; Lane 5: cooked meat emulsion; Lane 6: autoclaved meat emulsion; Lane 7: negative control.

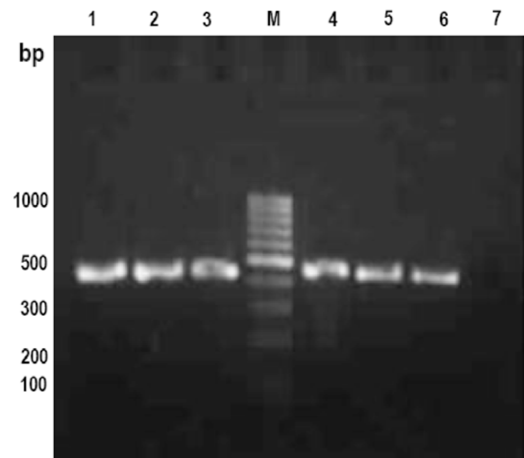

Fig. 5. PCR amplification of chicken mitochondrial D-loop gene with raw, cooked and autoclaved meat and meat emulsion admixed @ 1%. Lane M: 100 bp ladder; Lane 1: raw meat; Lane 2: cooked meat; Lane 3: autoclaved meat; Lane 4: raw meat emulsion; Lane 5: cooked meat emulsion; Lane 6: autoclaved meat emulsion; Lane 7: negative control.

positive reaction was also observed in quail and turkey. In-spite of positive reaction in turkey and quail, this primer pair was found to be very informative for differentiation of chicken from other meat species. This study reveals the importance of species-specific PCR assay in detection of species origin of meat and meat products even in admixed meat products. This species-specific PCR assay approach was also used earlier for identification of pork (Calvo, Osta, & Zaragoza, 2002), beef (Piknova & Kuchta, 2002), ostrich (Colombo, Viacava, & Giaretti, 2000) and mutton, chevon and chicken (Irfan-Ilhak & Arslan, 2007; Mane et al., 2007) in admixed meat products. This is one of the valuable tools to identify the meat species in presence of other meat species DNA (Hopwood et al., 1999).

The optimised PCR assay was further evaluated successfully for its efficiency to amplify the DNA extracted from fresh meat, cooked meat, autoclaved meat, raw meat emulsion, cooked meat emulsion and autoclaved meat emulsion of chicken. The PCR assay was found to be efficient to amplify the 442 bp DNA fragments from DNA extracted from heat treated chicken meat and meat emulsion (Fig. 4). No adverse effect of heat treatment, processing conditions and ingredients used for emulsion preparation was noticed on PCR amplification. Further, even autoclaving of meat emulsion at 121 °C, 15 psi for 15–20 min showed no effects on PCR amplifications. As mentioned in the earlier discussion, this may be due to heat stability and large number copies of mitochondrial DNA in meat tissue contributing to the survival of sufficient number of DNA copies, even when subjected to extreme processing conditions of autoclaving (Girish et al., 2004). Earlier, Hird et al. (2004) successfully applied chicken and turkey specific primer pairs based on mitochondrial cytochrome b gene for amplification of template DNA isolated from raw, boiled and autoclaved chicken and turkey meat. The effect of different cooking methods was also evaluated earlier for PCR amplification of mitochondrial DNA extracted from meat and meat products without any adverse remarks, except in pan-fried meat (Arslan et al., 2006). Kesmen, Sahin, and Yetim (2007) successfully employed the species-specific PCR assay for identification of meat species present in cooked meat sausages. They reported no adverse effect of processing conditions and ingredients used for preparation of cooked sausages on PCR amplification.

This PCR assay was subsequently tested for its sensitivity to detect the level of chicken in admixed minced meat and meat emulsion (containing beef, buffalo meat, pork, chevon, mutton) at 10%, 5%, 1% and 0.1% under different processing conditions. PCR amplification with specific band of 442 bp was detected in admixed minced meat having less than 1% adulteration (Fig. 5).

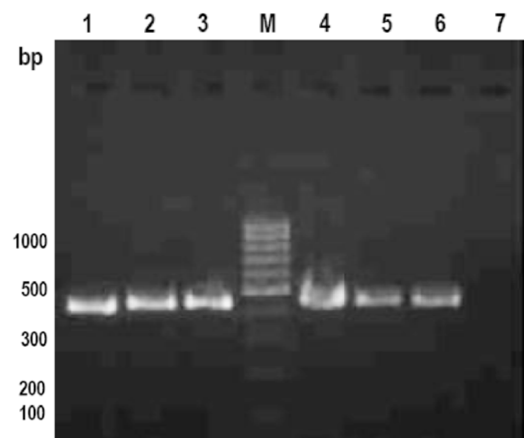

Fig. 6. PCR amplification of chicken mitochondrial D-loop gene with emulsion based meat products admixed @ 5% and 1%. Lane M: 100 bp ladder; Lane 1 and 4 @ 5% level: kabab; Lane 2 a0nd 5 @ 1% level: patty; Lane 3 and 6: block; Lane 7: negative control.

Finally, this PCR assay was successfully employed for detection up to 1% level of adulteration of chicken meat in admixed meat products (Patty, Kabab and block) (Fig. 6). These results clearly revealed that species-specific PCR is sensitive and specific assay for rapid identification of very low percentage of meat adulteration, even in meat products subjected to different processing conditions. Earlier, Calvo et al. (2002) also successfully developed swine-specific primers for detection of pork in wide range of meat and meat products in raw and cooked meats, sausages, cured meat products, hamburgers and pates. Hopwood et al. (1999) also developed and evaluated species-specific PCR assay for identification chicken in meat admixtures with detection of chicken, even in cooked meat admixtures at less than 1% level of adulteration in presence of other meats species such as beef, pork, lamb, horse, pheasant and duck.

4. Conclusions

The species-specific PCR assay was found to be precise, sensitive and rapid methods for routine analysis of meat species, even in ad-

mixed meat and meat products under different processing conditions. Thus, it can be concluded that the species-specific PCR assay can be useful tool for routine assessments of authenticity and quality of meat and meat products to protect the consumers from fraudulent practices of meat substitution.

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