



Sensitivity of Multiplex PCR Assay for Selected Animal Species

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ABSTRACT

Multiplex PCR assay was quick, simple and sensitive method for identification multiple meat species in single PCR reaction. Mitochondrial cytochrome b gene was used as an indicator gene for identification of selected animal species (Buffalo, Sheep, Goat and Chicken) by using multiplex PCR. Sensitivity of the multiplex PCR assay was determined by using 100 ng/μl DNA of meat of Buffalo, Sheep, Goat and Chicken. For the estimation of sensitivity of multiplex PCR assay (34 cycles) made subsequently 10 fold dilution of 100 ng/μl DNA i.e. 100, 10, 1, 0.1, 0.01, 0.001 ng/μl and used in multiplex PCR reaction. In present study the lowest sensitivity of the multiplex PCR assay for all the four selected animal species DNA concentration was fined up to 0.01 ng/μl.

Keywords: Multiplex PCR assay, primer mixture, sensitivity, cytochrome b gene

Adulteration of high cost meat with low cost meats of different origin sale of raw meat and preparation of meat products is a common practice in numerous countries. Since meat contamination and mislabelling are illegal and raise many health, religious and economic problems (Wang *et al.*, 2010), the detection of adulterations and quantity adulterants in meat products is crucial for the enforcement of labelling legislation and prevention of unfair competition (Kesmen *et al.*, 2010). Now a day different DNA-based methods used for animal species identification include DNA hybridization (Baur *et al.*, 1987; Janssen *et al.*, 1998), polymerase chain reaction (PCR) and its variants (Matsunaga *et al.*, 1999), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Girish *et al.*, 2005), random amplification of polymorphic DNA- polymerase chain reaction (RAPD-PCR) (Sebastio *et al.*, 2001; Mane *et al.*, 2006), PCR-sequencing and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) (Jurgen *et al.*, 2001). However, multiplex PCR assay was found to be quick and rate operative for identification of meat species

and successfully used for identification of several species of meat (Ilhak and Arslan, 2007).

The present study was planned for determination of the sensitivity of multiplex PCR assay for meat from buffalo, sheep, goat and chicken.

MATERIALS AND METHODS

In the present study meat samples of buffalo, sheep, goat and chicken randomly collected from the meat markets (15 samples from each species 50 mg and from muscles) at Palanapur, Gujarat in January, 2015. The mitochondrial DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions. The quality and purity of DNA were checked and quantification done by Pico-drop spectrophotometry and agarose gel electrophoresis. DNA samples showed 1.7 to 2.0 values for OD ratio (260 nm/280 nm) were considered of good quality and was used in the present study. In present study 100 ng μL⁻¹ DNA was used for sensitivity multiplex PCR. For the determination of sensitivity multiplex PCR made

subsequently 10 fold dilution of 100 ng μ L⁻¹ DNA *i.e.* 100, 10, 1, 0.1, 0.01, 0.001 ng μ L⁻¹ and used in multiplex PCR reaction as a DNA template.

A set of primers specific to *cyt b* gene family (Matsunaga *et al.*, 1998; Jain *et al.*, 2007), was custom synthesized at sigma Aldrich. The primer pair used in the present study consisting of common forward primer SIM (5'- CCT CCC AGC TCC ATC AAA CAT CTC ATC TTG ATG AAA-3') and reverse primers, goat primer (5'-CTC GAC AAA TGT GAG TTA CAG AGG GA-3'), chicken primer (5'-AAG AT A CAG ATG AAG AAG AAT GAG GCG-3'), buffalo primer (5'-CTA GAA AAG TGT AAG ACC CGT AAT, ATAAG-3'), sheep primer (5'- CTA TGAATG CTG

TGG CTA TTG TCG CA-3'). Initially species specificity of primers was tested by conventional PCR using a pair of the common forward primer and the species specific reverse primers with DNA of meat of selected animal species.

After conforming of species specificity of each primer multiplex PCR was carried by mixing of primers in the different ratio *viz.*, 2.5:1.5:1.5:1.5:3 for forward: Goat: Sheep: Chicken and buffalo specific reverse primers. Multiplex PCR reactions were carried out in a final volume of 25 μ L containing 3 μ L DNA template, 2 μ L primer mix, 12.50 μ L PCR master mixes and 7.50 μ L DNase-RNase free water. Multiplex PCR cycling protocol included

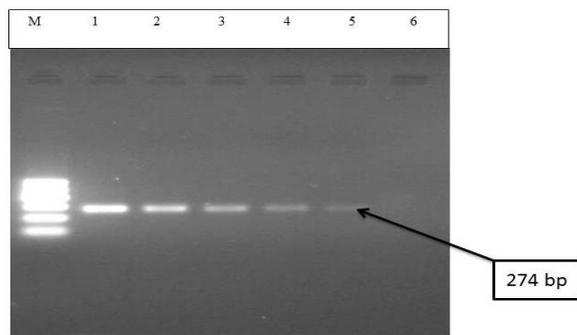


Fig. 1: 4% agarose gel electrophoresis of PCR products from the *cyt b* gene of buffalo meat. M: Marker 100 bp ladder; Dilution of DNA in 10 Fold (1,2,3,4,5,6); 1(100ng/ μ l); 2 (10ng/ μ l); 3 (1 ng/ μ l), 4 (0.1 ng/ μ l); 5 (0.01 ng/ μ l), 6 (0.001 ng/ μ l); Sensitivity of PCR assay from buffalo meat DNA was 0.01 ng/ μ l.

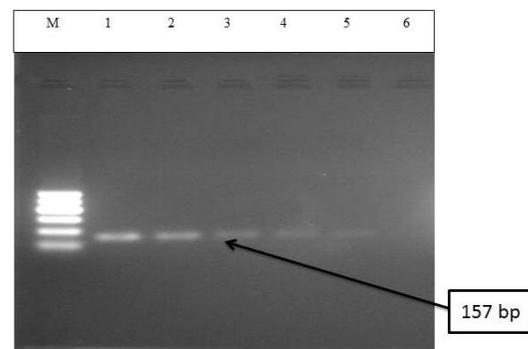


Fig. 2: 4% agarose gel electrophoresis of PCR products from the *cyt b* gene of goat meat. M: Marker 100 bp ladder; Dilution of DNA in 10 Fold (1,2,3,4,5,6); 1(100ng/ μ l); 2 (10ng/ μ l); 3 (1 ng/ μ l), 4 (0.1 ng/ μ l); 5 (0.01 ng/ μ l), 6 (0.001 ng/ μ l); Sensitivity of PCR assay from goat meat DNA was 0.01 ng/ μ l.

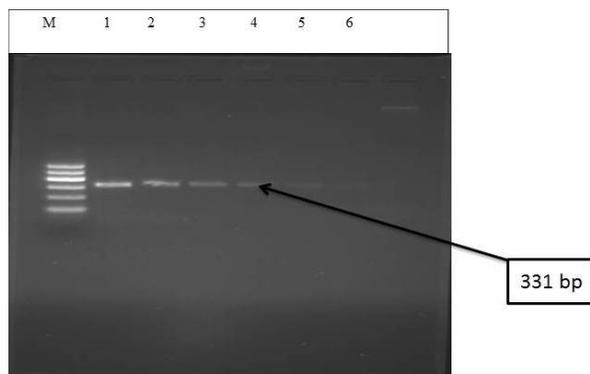


Fig. 3: 4% agarose gel electrophoresis of PCR products from the *cyt b* gene of sheep meat. M: Marker 100 bp ladder; Dilution of DNA in 10 Fold (1,2,3,4,5,6); 1(100ng/ μ l); 2 (10ng/ μ l); 3 (1 ng/ μ l), 4 (0.1 ng/ μ l); 5 (0.01 ng/ μ l), 6 (0.001 ng/ μ l); Sensitivity of PCR assay from sheep meat DNA was 0.01 ng/ μ l.

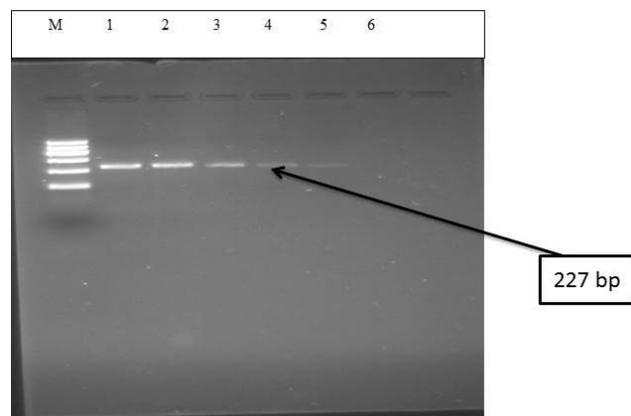


Fig. 4: 4% agarose gel electrophoresis of PCR products from the *cyt b* gene of chicken meat. M: Marker 100 bp ladder; Dilution of DNA in 10 Fold (1,2,3,4,5,6); 1(100ng/ μ l); 2 (10ng/ μ l); 3 (1 ng/ μ l), 4 (0.1 ng/ μ l); 5 (0.01 ng/ μ l), 6 (0.001 ng/ μ l); Sensitivity of PCR assay from chicken meat DNA was 0.01 ng/ μ l

initial denaturation at 94°C for 5 min. then followed by 34 cycles of 94°C for 30 seconds, 62°C for 30 seconds 72°C for 30 seconds and final extension at 72° for 10 min.

Multiplex PCR amplicons were resolved by 4% agarose gel electrophoresis. The 5 µL PCR products mixed with 1µL 6X gel loading dye were loaded @ 6 µL per well on 4 % agarose gel and electrophoresed at 80 V for 30 min using 0.5X TAE buffer.

RESULTS AND DISCUSSION

The sensitivity of multiplex PCR assay was determined by using 100 ng/µl DNA concentrations of all four selected animal species estimated by Pico-drop's Spectrophotometer (UNICAM). Agarose gel electrophoresis of PCR products from the *cyt b* gene of all four selected animal species (Buffalo, sheep, goat and chicken) up to 5th well, therefore the lowest sensitivity of multiplex PCR assay for Buffalo, sheep, goat and chicken DNA was fined up to 0.01 ng/µl respectively seen in figure 1,2,3, and 4.

In the present study, the multiplex PCR assay (34 cycles) sensitivity was found up to 0.01 ng/µl for all the DNA samples of various species. Our results was supported Nagapp, (2008). They found sensitivity up to, 0.1 pg/µl, 0.1 pg/µl, 1 pg/µl, 10 pg/µl, 10 pg/µl and 10 pg/µl in cattle, goat, sheep, buffalo, pig and chicken DNA, respectively. They found sensitivity 10 times lower than that observed in the present study (Gupta *et al.*, 2012). Noticed sensitivity of multiplex PCR assay (35 cycles) up to 0.25 ng/µl in goat, chicken, cattle, sheep, pig and horse DNA. They found level of sensitivity was approximately 25 times greater than observed in the present study by Matsunaga *et al.* (1999). The inequality of results originate in present study and to that of previous reports might have been obtained due to the type of meat piece, age of animals, sample size, Number of PCR cycles, etc. However, the present results suggest that even in the concentration of 0.01 ng/µl of DNA can be sufficiently detected in any meat samples.

CONCLUSION

In present study determination of sensitivity multiplex PCR assay made subsequently 10 fold dilution of 100 ng/µl DNA *i.e.* 100,10, 1,0.1,0.01,0.001 ng/µl and used in multiplex PCR reaction as a DNA Template. Initially

species specificity of each primer pair was confirmed by conventional PCR. After confirmed species specificity of each primer a multiplex PCR was designed by mixing all primers in a single reaction multiplex PCR assay was successfully amplified for DNA of meat samples of goat, chicken, buffalo and sheep. In present study the lowest sensitivity of the multiplex PCR assay for all selected animal species DNA concentration was found up to 0.01 ng/µl.

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