SHORT REPORT
doi: 10.2306/scienceasia1513-1874.2011.37.170

ScienceAsia 37 (2011): **170–173**

Single-nucleotide primer extension assay of mtDNA to authenticate cattle and buffalo meat

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Received 6 Nov 2010
Accepted 18 May 2011

ABSTRACT: A reliable method for identifying and differentiating cattle and water buffalo meat by fluorescence-based single-nucleotide primer extension assay is presented. The method used genotyping (single nucleotide primer extension) of the *cyt b* gene region of mitochondrial DNA extracted from meat samples. Meat samples were subjected to different levels of autolysis to simulate various meat processing technologies. The assay indicates absolute specificity and a high sensitivity, even to low DNA concentrations of 0.01 pg (1%) of adulteration in cattle-buffalo meat mixture, and can be applied with equal efficiency to fresh, cooked, or putrefied meat.

KEYWORDS: meat authentication, genotyping

INTRODUCTION

Efficient assays to precisely and rapidly detect the origin of meat are always in demand. Identification of the meat origin from processed meat products is a current matter of concern for a variety of economic, religious, and health reasons. The extensive development of nucleic acid based technologies over the past decade reflects their importance in food analysis. Various PCR based approaches have been attempted for meat authentication, but only a limited number of studies targeted buffalo as one of the species under study. In recent years, methods have been developed that use polymerase chain reaction (PCR) coupled with techniques such as hybridization, nucleotide-sequencing, single-strand conformation polymorphism, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism, or forensically informative nucleotide sequencing to differentiate water buffalo meat from cattle meat [1–7].

The application of fluorescence-based assay formats greatly simplifies protocols for DNA detection and overcomes the limitation and ambiguities of the PCR-based detection. The disadvantage of heteroduplex and single-strand conformation polymorphism analyses is that confirmatory testing (usually by sequencing) is required for all positive results to definitively identify the sequence variant, but the single-nucleotide primer extension (SNaPshot) assay is a very specific assay, producing sequence data for which confirmatory testing is not required [8]. Recently, SNaPshot assay has been used to authenticate meat from game and domestic species [9, 10]. The present study describes a rapid and reliable method to identify and differentiate cattle and water buffalo meats targeting the mitochondrial *cyt b* region using a SNaPShot assay.

MATERIALS AND METHODS

Sample preparation and DNA isolation

Thirty fresh muscle meat samples for each species were collected from the local slaughterhouse and stored at −20 °C until use. Meat samples (3 g) from each species were minced and mixed at various proportions, divided into five replicates, and subjected to various experimental procedures of cooking and putrefaction. Meat samples were cooked at 100 °C and 120 °C in a dry hot air oven and in moist heat (water bath and autoclave) for 45 min to simulate various methods of cooking. Often meat samples are brought to the laboratory for speciation after one or two days after slaughter under unpreserved conditions. Therefore different levels of autolysis were produced by allowing the meat samples to putrefy for a variable duration (48 h to 72 h) at room temperature in unpreserved conditions to stimulate the autolysis in meat. Mitochondrial DNA (mtDNA), along with genomic DNA was extracted by using a method described in Ref. 11. The DNA quantity and quality was estimated by measuring the A_{260}/A_{280} ratio using

Fig. 1 Nucleotide sequences of the target region and primer locations on the water buffalo and bovine *cyt b* gene. Primer positions are underlined and target nucleotides are shown in bold. Dots indicate identical nucleotides in both sequences.

a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA).

Polymerase chain reaction

A small fragment of *cyt b* gene of mtDNA extracted from each of the fresh, cooked, and putrefied samples was amplified. For this purpose a SNaPshot primer (genotyping primer) was designed by modifying the reverse primer (5′-$(T)_{12}$GGCATTGGCTGAATGGC-CGGA A-3′), as described in Ref. 12 and the forward primer was taken as such (Fig. 1). The water buffalo *cyt b* gene sequence[13] (gene bank accession number D 32193) was compared with the bovine *cyt b* gene sequence[14] to choose the most suitable region for designing the primers. A 22 nucleotides length genotyping primer was elongated by adding 12 thymidine nucleotides at the 5′-end, making it reasonably long to be resolved by capillary electrophoresis[15]. PCR was performed in a Master Cycler gradient thermocycler (Eppendorf, Germany). The total PCR solution volume of 25 µl contained 1× PCR master mix (MBI Fermentas, Canada), 10 pmol of each primer, and 50 ng of DNA template (cattle and/or buffalo). The PCR reaction was performed under the following cycling conditions: an initial heat denaturation at 95 °C for 5 min; 35 cycles as follows: 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 5 min. Amplified products were confirmed by using Genesnap and Genetool (Syngene, UK), and running the products parallel to a 100 bp MW marker. After amplification, PCR products were purified to remove primers and unincorporated dNTPS by Perfectprep PCR clean-up plate (Eppendorf, Germany) according to the manufacturer's protocol.

SNaPshot assay

The SNaPshot assay was performed in a Master Cycler gradient thermocycler (Eppendorf, Germany) as per the manufacturer's protocol. The SNaPshot reaction consisted of 5 µl of SNaPshot ready reaction mix (ABI), 0.01–0.4 pmol of purified PCR products, 2 mM genotyping primer in the final reaction, and nuclease-free water to a final reaction volume of 10 µl. The amplification consisted of 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 30 s.

After the SNaPshot reaction, a post extension treatment to remove the 5′- phosphoryl group of the ddNTPs helps to prevent unincorporated terminators from comigrating with the extended primers and producing a high background signal. For this, a 10 µl final volume was treated with 1 µl of CIP (MBI Fermentas, Canada) for 60 min at 37 °C, followed by 15 min at 85 °C for enzyme inactivation. Then, 1 µl of the SNaPshot products was mixed with HiDi formamide and LIZ 120 (Applied Biosystems) size standard and denatured at 95 °C for 5 min, and then placed on ice for at least 2 min. The fluorescently labelled fragments were resolved by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) for 24 min at 15 kV and 60 °C using a GS POP-4 (1 ml) E5 control module. The resulting data in the form of colour, position of peak, and area under the curve was analysed by GeneMapper 3.7 (Applied Biosystems).

RESULTS AND DISCUSSION

The processing technology applied during the manufacture of meat products affects the integrity of the extractable DNA causing its degradation into small fragments. This is more valid for nuclear DNA and in these cases, use of small fragments of DNA has been recommended[16,17]. For these reasons, short amplicons of 152 bp were amplified from the *cyt b* gene of mtDNA. In general, mtDNA targeted methods predominate in such studies because of the high copy number of small, circular mtDNA in cells which ensures a sufficiently high quantity of PCR product, even when small amounts of fresh or processed tissues are used[6]. PCR amplified 152 bp fragments for both cattle and buffalo samples (fresh, cooked, and putrefied). Amplification or cross-reaction was not observed when DNA samples of sheep, goat, pig, horse, or chicken were used (data not shown).

SNaPshot requires an unlabelled genotyping primer that anneals to the PCR product template immediately upstream of the target SNP on the template. The reaction contains only dideoxynucleotide (ddNTP) terminators (no dNTPs) such that during thermal cycling, the DNA polymerase can extend the primers by only one base (ddNTP), which is complimentary to the template strand at the site of interest

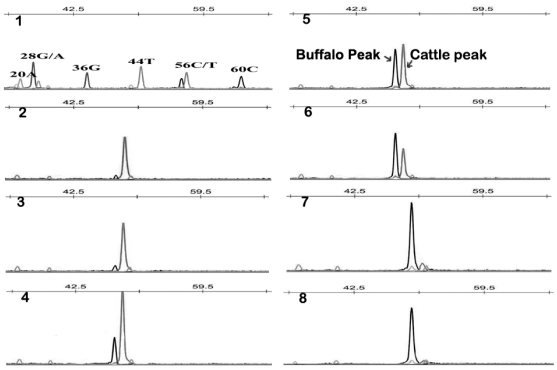

Fig. 2 Electropherograms: x-axis is size in bases, y axis is fluorescence intensity. SNaPshot products resulting from extension of the genotyping primer: left black peak for buffalo or/and right grey peak for cattle. Lane 1: control sample provided with SNaPshot Kit, Lanes 2–8 are cattle and buffalo mixed samples in the ratio (cattle: buffalo) of 99:1, 90:10, 75:25, 50:50, 25:75, 10:90, and 1:99, respectively.

such that the products can be separated, sized, and detected by capillary electrophoresis. Each ddNTP is labelled with a different fluorescent signal/fluorochromes (A-dR_6G: green, G-dR110: blue, T dRox: red, C-dTAMRA: yellow) allowing for the detection of all four nucleotides in the same reaction.

In the present study, genotyping primer gave a peak for cattle DNA (as Rox-ddT incorporated) and the same gave a peak for buffalo DNA (as dTAMRA-ddC incorporated). The same concentrations of cattle and buffalo DNA templates produced different peak heights depending on the terminator and dye label. In general, thymidine labelled with dRox (6-carboxy-x-rhodamine) produces a higher signal than cytosine labelled with dTAMRA (N, N, N', N'-tetramethyl-6-carboxyrhodamine). Some fragments display a stronger fluorescent signal than others in the electropherogram, even after colour compensation with the appropriate matrix, due to variation in SNaPshot chemistry. However, this fact does not unduly affect the readability of the electropherogram[18]. Quantification was possible as the primer set hybridized at the homologous position in the cattle and buffalo DNA with the same annealing affinity and amplified the DNA fragment of identical size.

To measure the sensitivity of the presented SNaPshot reaction, 100 ng of DNA sample was 10-fold serially diluted and tested to observe positive signals up to 0.1 pg. The sensitivity and discriminating power was also evaluated by serial dilution of mixed meat samples (cattle:buffalo). SNaPshot was found to be capable of detecting 1% adulteration in cattle-buffalo meat mixture tested (Fig. 2). On further dilution the signals ceased abruptly. The assay was not tested for better sensitivity, which cannot be ruled out. False positive and false negative results were not encountered in this assay, demonstrating the reliability of the procedure. Repeated assays proved the reproducibility of the method. Detection of adulteration from degraded DNA obtained from cooked and putrefied samples is one of the very important merits of this technique as a tool for meat species detection.

The SNaPshot assay described here provides a very sensitive and specific assay to identify and differentiate cattle and buffalo meat. This method can be applied with equal efficiency to fresh, cooked, and putrefied meat. However, the method is aimed at traceability and verification and as such needs to be tested in blind meat mixtures or commercial meat products to validate its accuracy in a real-life situation.

Acknowledgements: This study was supported by the Department of Biotechnology, Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat. We also thank Dr C.D. Bhong for his help with obtaining various known and unknown meat samples from the slaughterhouse for use in the study.

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