DOES DURATION OF COOKING HAVE AN EFFECT ON PCR DETECTION OF MEAT FOR FORENSIC PURPOSES?

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ABSTRACT

Although conventional Polymerase Chain Reaction (PCR) is one of the best methods used for meat identification for forensic purpose, some samples of cooked meat presented to Veterinary Research Institute for meat species identification have not responded in conventional PCR. Objective of this research was to exclude one of the possible reasons that would have caused this problem. To identify the effect of different cooking time on Deoxyribo Nucleic Acids (DNA) extraction and PCR, beef was used as the meat type, since very often the suspicious sample is claimed to be 'beef'.

Total of 18 samples of beef from 3 different commercial sources were used. Samples (n=6) from each source were cut into equal sizes and cooked separately to minimize contamination. They were cooked at 20 min, 40 min and 60 min cooking periods by adding equal amounts of commercially available products of turmeric powder, curry powder, chillie powder, salt powder and water. Samples were kept separately until DNA extraction. Forward and reverse primers were used for DNA amplification of bovine cytochrome b gene. The samples were subjected to DNA quantification by using the nanodrop spectrophotometer. Change in absorbance by DNA samples was used to quantify the DNA samples.

The results of gel electrophoresis revealed that the samples were positive in all 3 cooking conditions with bands of ~ 272 bp equivalent compared to ladder and the positive control sample. Statistical analysis of DNA quantities revealed that even though the cooking time (up to 60 min) had no effect on the extracted DNA for species identification of beef samples as mentioned above, the DNA samples extracted from beef samples at 60 minutes resulted in high absorbance values indicating possible denaturation and fragmentation of DNA.

Key words: *Meat identification, cooking time, PCR*

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INTRODUCTION

Taste and nutritional value makes meat a main component of human diet. In Sri Lanka, meat is supplied by government slaughter houses in Kandy and Colombo and Island wide slaughter houses maintained by the 'Pradeshiya Sabhas'. Some authorized private companies such as "Prima", and "Crysbro" etc. also supply meat to the market. Though the authorized bodies play a major role in supplying meat, 'bush meat' is also present in the market. Increase in bush meat and illegal killing of animals can be due to uncontrollable access to wild life, lack of education, poverty, unemployment etc.

Bush meat and the illegal slaughtering of animals have led to many problems in religious, legal, ethical, health and economic sectors of the country and also it is a threat to the wild life. In Sri Lanka, many wild animal species belonging to the 'Cervidae' family

are illegal to be killed. Ceylon spotted deer (*Axis axis ceylonensis*), Ceylon hog deer (*Axis porcius oryzus*), Ceylon Sāmbhar (*Cervus unicolor unicolor*) and barking deer(*Muntiacus muntjak malabaricus*) are considered as protected animals (Fauna and Flora protection Ordinance 1937; Rajapaksha *et al.,* 2003). Buffaloes (*Bubalus bubalis*) are also banned to slaughter in Sri Lanka according to the Animal act no 29 of 1958. In order to discourage illegal hunting, to protect wild life, and to avoid substitution of meat species, forensic identification of meat is important. Though there are regulations stipulated to conserve and protect animals and to prevent selling their meat, implementations of the legislations is limited due to the failures of identification of meat (Rajapaksha *et al.,* 2001).

In Sri Lanka meat samples are submitted by the court and the wild life department to the Veterinary Research Institute (VRI), Gannoruwa for species identification. Most common claim for the suspicious meat sold is 'beef'. If the laboratory findings do not prove that the received meat sample as 'beef' then specific species identification is required for further investigation. There are various methods used for identification of species origin of meat such as sensory analysis, anatomical variation of species, and histological differentiation of the hair on meat, tissue fat properties, level of glycogen in muscle tissue, electrophoresis and DNA hybridization. Methods such as Agar Gel Precipitation Test (AGPT), dot blot assay or counter immunoeletrophoresis are also being used for meat species identification (Dissanayaka *et al.*, 2001). Detection of the specific meat proteins, which may be denatured or destroyed during processing and process of rotting, is the basis of these methods. Other than that Polymerase Chain Reaction (PCR), Restricted Fragment Length Polymorphism (RFLP) and Random Amplified polymorphic DNA (RAPD) techniques are used for meat species identification (Ilhak and Arslan, 2006). Out of these numerous methods mentioned above, VRI uses conventional PCR for meat species identification. PCR easily amplifies the target regions of template DNA even in the presence of a small quantity within a much shorter time. When compared with protein, DNA is more stable and resistant to factors such as high temperature, chemicals and pressure. Because of these properties identification of different meat species including both raw meat as well as the meat subjected to thermal treatments is possible at present (Spychaj *et al.*, 2009).

However, the extraction of DNA for species identification of meat is not possible from all the samples presented to the laboratory which may be due to the nature of the samples. The majority of the samples received by lab are either rotten or processed (Rajapaksha et al., 2003). Some samples are stored and transported under different conditions and some are already cooked samples. Cooked samples can vary with cooking conditions depending on the duration of cooking (being overcooked, under cooked), cooking temperatures, amount of water and spices added etc. For an example usually curry powder, chillie powder, turmeric powder, salt are added during beef cooking. Other than that, sera (*Cymbopogoncitratus*), rampe (*Pandanusamaryllifolius*), ginger (*Zingiberofficinale*) and tamarind (*Tamarindusindica*) are added during meat cooking to increase flavor and moisture. Due to the effect of one or more of the factors mentioned above only some samples respond to the PCR while some do not.

Failure of species identification results in failures in administering justice. Though it is considered that the PCR technique is successful at identification of meat, lack of sensitivity of PCR assay for cooked meat of members of Cervus family was reported by Rajapaksha et al., 2002 and same was reported for boiled horse meat by Mastunaga et al., 1999.

Exclusion of the factors that do not influence on the DNA extraction of meat is useful in determining the sensitivity of the method and narrowing down the investigations on the possible causative factors for PCR failures.

Therefore, the aim of this study was to analyze the effect of time duration of cooking on the DNA quantity yield and its effect on the sensitivity of PCR by taking beef as a convenient sample. The study compared three different cooking times of 20min, 40 min and 60min with the DNA extraction of meat (beef).

MATERIALS AND METHODS

1. Sample Collection and Preparation

Fresh beef samples were obtained from 3 different commercial sources. Each Sample was washed properly and labeled as A, B, and C. Each sample was cut into 6 pieces each of approximately 2cm x 2cm x 2cm. The 6 pieces from each sample were put into separate clay pots. Following amounts of commercially available spices and water were added to each pot just before cooking.

Recipe:

*Curry powder	1 tsp
Chillie powder	1 tsp
Salt powder	½ tsp
Turmeric Powder	¼ tsp
Water	1 cup

*Consistency of curry powder: coriander, cumin, fennel, turmeric, cardamom, cloves, Fenagreek, Cinnamon, Rampe and curry leaves. Consistency of salt: Edible salt, KI
All ingredients were mixed well with meat pieces. The initial temperature was recorded at each cooking session. The samples were cooked in a closed container under relevant cooking conditions on a hot plate. Two pieces were taken out at the time intervals of 20 min as follows:

20 min- 2 pieces
40 min- 2 pieces
60 min- 2 pieces

The samples were wrapped in sealant bags and grouped according to the time duration of cooking and kept in the freezer at -20°C until DNA extraction.

2. DNA Extraction

Approximately 50 mg of each tissue sample was measured and the following procedure was adopted for each sample separately.

Each sample was crushed gently using a tissue grinder. Digestion buffer (600µl) was added to each sample placed in labeled eppendorf tubes.

Digestion buffer preparation

3M Nacl	800
1MTris-Hcl	250
0.5 M EDTA	1.25 ml
10% SDS	1.25 ml
(pH 8.4)	
Proteinase k enzyme	100µg/ ml

Filled up to 25 ml with distilled water.

The sample was incubated at 35^0C for 3 hrs. Equal volume (600µl) of Phenol: chloroform: iso amyl alcohol (25:24:1) in which pH was 7.9 was added to it. The sample was mixed well using vortex and centrifuged at 14000rpm for 5 minutes at room temperature. The supernatant was taken in to a separate tube and 60µl of 3M Nacl was added to each sample. Then 1.2 ml of Ethanol was added and vortexed well. The samples were kept at -20°C in freezer for an overnight. After that the samples were centrifuged at 14000rpm for 10 minutes at room temperature.

The supernatant was discarded and 120µl of 70% Ethanol was added to the remaining pellet, centrifuged at 14000 rpm for 5 minutes at room temperature, the supernatant was removed and pellets were air dried. 100 µl of DNA free H_2O was added and samples were kept at -20^0C until used.

3. PCR technique

Each of 20 samples (including positive and negative control samples) were prepared for the PCR as shown in table 1.

Table 1: PCR constituents

Constituent	Amount(µl)
PCR buffer	3
dNTP	0.6
P_3 Primer	1
P_6 Primer	1
Taq Polymerase	0.3
DNA	1
DNA free water	18.1

The samples were loaded into 500µl eppendorf tubes and the PCR was performed under following PCR conditions:

95 °C – 3 min (pre dwell)
95 °C – 1 min (denaturation)
55 °C – 1 min (primer annealing)
72 °C – 1 min (polymerization)
72 °C – 7 min (post dwell)

Mitochondrial cytochrome b sequence was amplified using following two forward and reverse primers. This was used because mitochondrial DNA is more stable under various conditions compared to nuclear DNA.
Forward:
5/GACCTCCCAGCTCCATCAAACATCT CATCTTGATGAAA3/
Reverse :
5/CTAGAAAAGTGTAAGACCCGTAAT ATAAG3/

4. Gel electrophoresis

Gel electrophoresis was performed to separate and visualize the amplified PCR products according to the size. To prepare the gel, 1.5 g Agarose and 150 ml of TBE buffer was added and kept in microwave oven for 2.5 min to dissolve. The liquid was allowed to cool inside a biosafety cabinet. 12µl of ethidium bromide was added, mixed and poured on a chilled tray. It was let to set for 40 min. Loading mix was prepared and loaded to relevant well.

Ladder	loading buffer	2µl
	Ladder	5µl
Sample	Loading buffer	2µl
PCR product		6µl

Electrophoresis was done on agarose gel at 100 V for 45 minute resulting gel was visualized using a UV trans illuminator. The results were compared with the DNA ladder.

DNA quantification and the statistical analysis of the data obtained were performed. DNA concentration can be assessed using methods such as absorbance, agarose gel electrophoresis, fluorescent DNA binding dyes, etc. Most common methods are using spectrophotometer and the agarose gel electrophoresis. An increase in absorbance at 260 nm of the DNA solution is because of the denaturation of double stranded DNA to single stranded DNA. This is due to the increase in DNA yield.

RESULTS

1. Results of Gel Electrophoresis

All the 18 samples, 6 in each time period (20 min, 40 min, 60 min) of 3 beef sources gave bands equivalent compared to that of ladder and the positive sample as shown in figure 1,2 and 3.

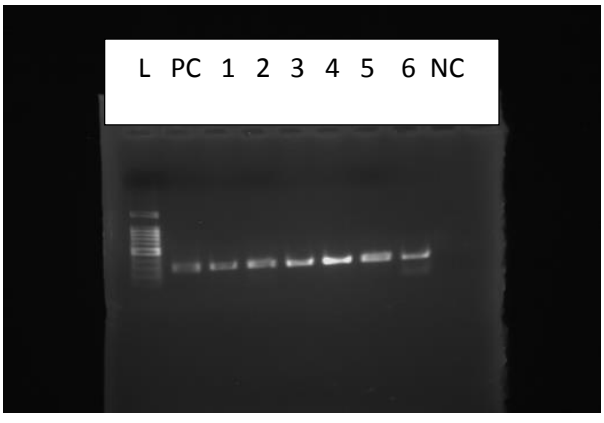

Figure 1 : Gel electrophoresis of the beef samples cooked for 20 minutes (PC: DNA extracted previously from meat and used as positive control, NC: Negative Control without DNA)

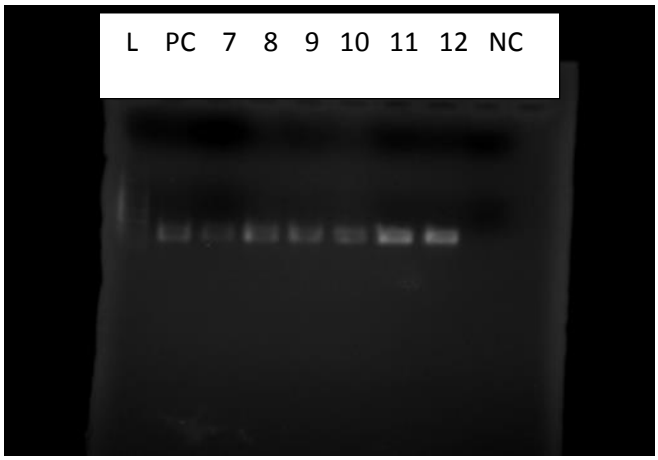

Figure 2 : Gel electrophoresis of the beef samples cooked for 40 minutes

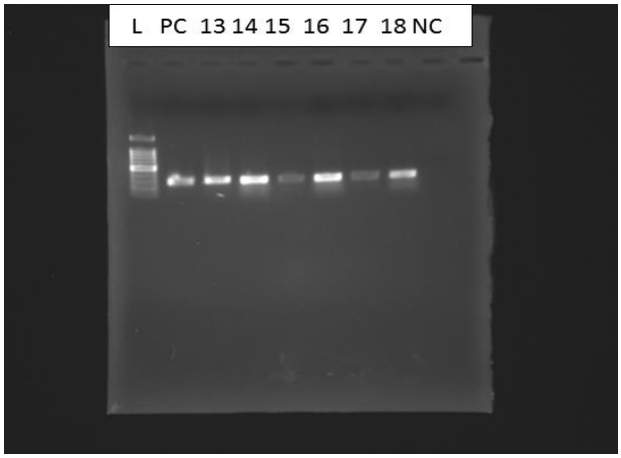

Figure 3: Gel electrophoresis of cooked beef samples at 60 minutes

When DNA was amplified a band of about 272 bp was observed for all the meat samples of different cooking time periods. Note that the bands of sample no 15 and of 17 were light (Figure 3). There was no band for negative control (NC; Figure 1, Figure 2, and Figure 3).

2. Quantification of DNA using nanodrop method

Table 2: Mean DNA quantities according to time

Cooking Time (min)		**DNA quantity (ng/µl)**		**Mean DNA quantity**
20	A_{20}	317.1	A_{20}	275.35
	A_{20}	233.6		
	B_{20}	583.5	B_{20}	340.25
	B_{20}	603.9		
	C_{20}	1050	C_{20}	1031.15
	C_{20}	1013		
40	A_{40}	559.9	A_{40}	961.45
	A_{40}	1363		
	B_{40}	732.9	B_{40}	1018.95
	B_{40}	1305		
	C_{40}	294.3	C_{40}	808.8
	C_{40}	1029		
	A_{60}	372.85	A_{60}	1557.42
	A_{60}	2742		
60	B_{60}	1414	B_{60}	2851
	B_{60}	4288		
	C_{60}	3280	C_{60}	2228
	C_{60}	1176		

3. Analysis of Data

Data obtained was analyzed using Minitab 15 software version to detect the effect of cooking time on the DNA quantity of the relevant samples.

One way ANOVA was performed to evaluate the difference of mean DNA quantities extracted according to the cooking time. The results indicated a statistical significance($p<0.05$) for the DNA quantity measured in terms of absorbance and this was mainly due to the difference observed in the mean absorbance for meat cooked for 60minutes compared to the means observed for 20min or 40min samples (Figure 4).

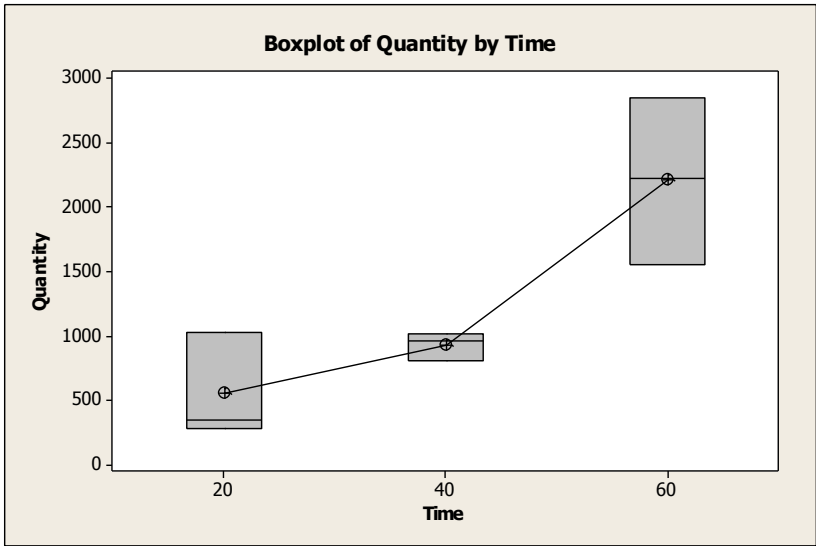

Figure 4: Box plot of DNA quantity according to cooking time

The box plot in figure 4 shows that there is less variation between the mean DNA quantities between samples cooked for 20 minutes and 40 minute, while the mean DNA quantity of the sample cooked for 60 minutes has a wide variation. Purity of the samples also was checked and contamination was ruled out.

DISCUSSION

As all the samples cooked up to 60 minutes gave bands in gel electrophoresis, successful DNA extraction (Figure 1, Figure 2 and Figure 3) was indicated and results indicate that conventional PCR method can be used to identify DNA extracted from the beef samples cooked up to 60 minutes. For the successful amplification of DNA purity and the quality of DNA template and the heating process has a greater impact.

The absorbance of DNA samples at Nano drop spectrophotometer indicates the DNA quantity. When the samples were cooked for longer time (60minutes) the absorbance of the DNA sample has been increased exponentially. This resulted in a statistically significant difference in the mean DNA quantity at 60minutes compared to 20minutes or 40minutes cooking time. This could be due to the release of more DNA from cells followed by denaturation and fragmentation of DNA released with prolonged cooking. The relatively high variation between the mean DNA quantity and lighter bands in gel electrophoresis of 60 minutes cooked beef sample may be due to the high temperature exceeding boiling temperature of water (100ºC) at the time of 60 minutes resulting in DNA fragmentation (Musto *et al.*, 2010). Caution is required when the fragments become smaller which affects the sensitivity of PCR technique. However up to 60minutes of cooking all the beef samples were able to produce a positive band at ~272bp level on the ladder.

CONCLUSION

PCR and gel electrophoresis can be used effectively to identify beef samples cooked for 20 min, 40 min and 60 min time in species identification of meat for forensic purpose. High mean quantity of DNA was yielded from samples cooked for 60 min when

compared with the quantity of DNA of 20 min and 40 min of cooking. This indicates possible DNA fragmentation at 60 minutes of cooking. However, this did not affect the sensitivity of the test. Therefore, it can be concluded that the species identification of beef is possible in conventional PCR method if they are cooked under the conditions as in this experiment and cooked up to 60 minutes.

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