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# Comparison of different DNA preparatory methods for development of a universal direct PCR-RFLP workflow for identification of meat origin in food products

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**Abstract**

The quality and quantity of the extracted DNA are two key aspects for a successful PCR (Polymerase Chain Reaction) amplification. Moreover, reduction in time and cost required for DNA extraction are also two considerable factors, in cases when large number of samples are to be analyzed within a limited time-frame and budget. Accordingly, the aim of this study was to compare and optimize performance of five different DNA extraction methods by boiling meat tissues from cattle, buffalo, sheep, goat, chicken, camel, horse and dog in PBS (Phosphate Buffer Saline), distilled water, alkaline lysis buffers 1, 2 or 3. The results indicated that the boiling of meat and its products in alkaline lysis buffers was a good method to extract crude DNA. The optimized crude DNA extraction protocol was coupled with PCR-RFLP (Restriction Fragment Length Polymorphism) analysis for meat species differentiation. This developed workflow was tested on fifty-three commercial beef and mutton samples, out of which three samples were found to be adulterated. In conclusion, the rapid crude DNA extraction protocol has led to the development of a direct PCR-RFLP workflow that is simple, time-saving and cost-effective for PCR-based identification of different meat species.

**Keywords:** DNA extraction; direct PCR-RFLP; meat adulteration; species identification.

**Practical Application:** An optimized direct PCR protocol coupled with Restriction Fragment Length Polymorphism for simple and time-saving identification and detection of the adulteration of different meat species.

## 1 Introduction

Meat is an essential part of human diet and is processed into various food commodities, such as meat patties, nuggets, and meatballs all around the world (Kang & Tanaka, 2018). Moreover, all the development that has taken place in the meat industry has also increased the incidents of meat adulteration and fraud for economical gain (Sheikha et al., 2017; Sheikha, 2019). Two of such meat adulteration incidents include horse meat scandal of UK (2013) and China's fake meat scandal of 2013 (Premanandh, 2013). Now days, food fraud has emerged as one of the major global issues (Mansouri et al., 2020). As a result, the search for rapid and more efficient meat species detection methods has quickened. Some of the recently developed techniques for meat species identification include high resolution melting curve analysis (Njaramba et al., 2021), single-tube multiplex PCR (Iqbal et al., 2020), digital droplet PCR (Yu et al., 2021), two-tube hexaplex PCR (Cai et al., 2022) and real-time quantitative PCR (qPCR) (Taniguchi et al., 2022).

It is well-acknowledged that extraction of genomic DNA is an imperative step to ensure amplifiable DNA template (Mokhtar et al., 2020). However, conventional DNA extraction protocols for molecular meat testing are complicated, labor-intensive, time-consuming and expensive (Besbes et al., 2022; Sajali et al., 2018; Yue & Orban, 2001). An alternative approach to increase the efficiency of meat speciation is by eliminating laborious and time-consuming DNA extraction steps and directly allowing the samples for amplification, termed as direct PCR (Schnepf et al., 2013). Direct PCR is rapid, eliminates the need for purification steps, and proves more sensitive than conventional PCR (Linacre et al., 2010; Swaran & Welch, 2012). Although previously reported, these methods either require special and expensive polymerases and extraction kits or have limited efficiency for amplification (Ben-Amar et al., 2017; Guan et al., 2019; Thanakiatkrai et al., 2019).

DNA extraction methods that use brief boiling of samples at high temperatures reduce time, labor and cost and have been demonstrated as an efficient DNA extraction technique in many studies (Kieleczawa, 2006; Alasaad et al., 2012; Kitpipit et al., 2014b). DNA extraction by boiling samples in alkaline lysis (AL) buffers has been successfully applied to amplify DNA samples from blood, feathers and many other tissues (Truett et al., 2000; Haunshi et al., 2008; Girish et al., 2013), but the different modifications/compositions of AL buffers have never been compared for their DNA extraction potential to identify vertebrate species in meat and meat products. The AL technique holds advantage over conventional DNA extraction methods as it is a simple, and rapid (10-30 min) procedure that requires

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minimum laboratory equipment and reagents (Ali et al., 2017; Zieritz et al., 2018; Girish et al., 2020; Mounika et al., 2021; Zhao et al., 2021). Moreover, scientists have also utilized distilled water and PBS as boiling buffers for DNA extraction (Sepp et al., 1994; Truett et al., 2000). With the escalating cases of food mislabeling and adulteration, the need for an easy, effortless and cost-effective DNA extraction method has intensified. It is thus imperative to compare and optimize these DNA extraction protocols to identify the most potent, rapid, less laborious, and cost-effective method that could make meat speciation from raw and cooked food easy and affordable.

Therefore, in the first step of this study we investigated the potential of five previously reported boiling DNA extraction methods for a direct PCR approach. These methods mainly differ in the buffer used for sample processing i.e. PBS, distilled water, alkaline lysis buffers 1, 2 and 3. Upon initial screening we found that PBS and distilled water do not provide detectable results at 10 min boiling duration and thus, alkaline lysis buffers 1, 2 and 3 were selected for further testing. The selected protocols were tested on the basis of their sensitivity, specificity, and reproducibility using meat samples from eight species including five most commonly consumed meat species (cattle, buffalo, sheep, goat and chicken) and three possible adulterant species (camel, horse and dog).

As the main goal of this study was to identify a simple workflow that would make meat speciation easier and affordable, in a second step, we developed a simple PCR-RFLP assay to discriminate the eight targeted meat species because it distinguishes different species from meat mixtures with the help of a single pair of universal primers and a restriction endonuclease (Murugaiah et al., 2009), without the need of sequencing. To the best of our knowledge, no previous study has compared the performance of different crude DNA extraction methods for the development of a direct PCR-RFLP analysis for identification of meat origin in food products.

## 2 Materials and methods

### *2.1 Sample collection and preparation*

Raw authenticated muscle tissue samples of eight species: Cow (*Bos indicus* Linnaeus, 1758), Buffalo (*Bubalus bubalis* Linnaeus, 1758), Sheep (*Ovis aries* Linnaeus, 1758), Goat (*Capra hircus* Linnaeus, 1758), Chicken (*Gallus gallus* Linnaeus, 1758), Camel (*Camelus dromedarius*), Horse (*Equus caballus* Linnaeus, 1758), and Dog (*Canis lupus familiaris* Linnaeus, 1758), were collected from the postmortem section of Department of Pathology, University of Veterinary and Animal Sciences Lahore (Punjab, Pakistan). Additionally, samples of whole, ground, processed, and uncooked beef and mutton were collected from the local markets of Lahore for the validation of the proposed assay. Commercially cooked food samples were also purchased from local restaurants to test the applicability of the developed procedure.

Meat pieces from each collected sample were washed with distilled water to remove blood and visible impurities in clean petri plates. Sterile blades were fixed every time on the scalpel for each specimen to avoid cross contamination while cutting the meat into small pieces. 70% ethanol was sprayed, left for 1 to 2 min and then decanted to remove any type of contamination. Small cut portions of meat were again washed by distilled water to remove the ethanol residues which may act as an inhibitor in the amplification step. The decontaminated and washed portions were further homogenized with the help of blade in the petri plate and all the homogenized samples were preserved at -20 °C immediately to avoid DNA degradation until needed for DNA extractions.

### *2.2 DNA preparation*

Reference meat samples were subjected to DNA preparatory methods for direct PCR by boiling with five different buffers i.e. water (Sepp et al., 1994), PBS (Kitpipit et al., 2014b), alkaline lysis buffer 1 (Girish et al., 2013), alkaline lysis buffer 2 (Tagliavia et al., 2016) and alkaline lysis buffer 3 (Truett et al., 2000). To achieve maximum positive results the recipe of each buffer was followed as described by the reported protocols. The steps followed for DNA preparation by each buffer are shown in Table 1.

For comparison of the DNA extraction efficiency of each buffer, three types of experiments were conducted:

- By varying the amount of meat sample taken for extraction (10 mg, 25 mg, 50 mg)

**Table 1.** Scheme for DNA preparation for direct PCR using five different boiling methods.

| Sr. No. | Step 1 | Step 2 | | | Step 3 | | Step 4 | References |
|---|---|---|---|---|---|---|---|---|
| | Eppendorf tube labeling | Boiling solution | Volume | Boiling duration at 100 °C | Neutralization buffer | Volume | Centrifugation at 4 °C & 4000 rpm | |
| **1** | A (10 mg meat sample) | Phosphate Buffer Saline (1×) | 150 µL | 10 minutes | None | None | 5 minutes | Kitpipit et al. (2014b) |
| **2** | B (10 mg meat sample) | Distilled water | 150 µL | 10 minutes | None | None | 5 minutes | Sepp et al. (1994) |
| **3** | C (10 mg meat sample) | 0.2N NaOH | 75 µL | 10 minutes | 0.04M Tris-HCl (pH = 7.75) | 75 µL | 5 minutes | Girish et al. (2013) |
| **4** | D (10 mg meat sample) | 200 mM KOH, 2 mM $Na_2EDTA$, 0.2% Triton X-100 | 75 µL | 10 minutes | 100 mM Tris-HCl | 75 µL | 5 minutes | Tagliavia et al. (2016) |
| **5** | E (10 mg meat sample) | 25 mM NaOH, 0.2 mM $Na_2EDTA$ (disodium EDTA) | 75 µL | 10 minutes | 40 mM Tris-HCl pH (5) | 75 µL | 5 minutes | Truett et al. (2000) |

- By varying the boiling (100 °C) durations at which a sample was boiled in buffer (2.5 min, 5 min, 7.5 min, 10 min, 20 min and 30 min)
- By varying the overall volume of the buffer added to the sample (150 µL, 200 µL and 500 µL)

After initial comparison, alkaline lysis buffers 1, 2, and 3 were selected for further studies on the basis of their efficiency. The comparison and optimization of alkaline lysis buffers 1, 2 and 3 were conducted in three sets of experiments. Firstly, 50 mg sample was boiled in 500 µL buffer, second was 25 mg sample boiled in 200 µL buffer and third was 10 mg sample boiled in 150 µL buffer. All three experimental sets were subjected to four different boiling durations (10 min, 7.5 min, 5 min, 2.5 min) and DNA template used for direct PCR was varied to four different volumes (2 µL, 1 µL, 0.5 µL and 0.25 µL).

### *2.3 Primer designing*

Already reported nucleotide sequences for mitochondrial 16S rRNA gene were downloaded from NCBI nucleotide database and aligned using Clustal platform in MEGA X (Kumar et al., 2018) for designing primers. A novel universal set of primers (Fd: 5'- AAGACGAGAAGACCCTGTGGAGCTT-3'; RC1: 5'-CGGTCTGAACTCAGATCACGTAGG-3') enclosing a fragment of ~317 bp was selected from highly conserved regions of the 16S rRNA gene sequences. Primers were picked arbitrarily according to the conditions described by Riaz et al. (2011) and were validated by OligoCalc: an online Oligonucleotide Properties Calculator (Kibbe, 2007).

### *2.4 PCR amplification*

The parameters for PCR amplification were optimized by varying the concentration of $MgCl_2$, primers, *Taq* DNA polymerase and the temperature for primer annealing. The finalized PCR reagents recipe was followed by mixing a range (0.5 µL, 1 µL and 2 µL) of template DNA, 2.5 µL (10×) *Taq* buffer, 1.5 µL (25 mM) $MgCl_2$, 2 µL (2.5 mM) dNTPs, 0.5 µL (10 pmoles) forward primer, 0.5 µL (10 pmoles) reverse primer, 0.25 µL (1.25 U) *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and water to maintain the final volume of 25 µL. The touch down PCR was performed for all reactions with following conditions: an initial denaturation at 95 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 30 secs, annealing at 63 °C for 30 secs (1 °C reduction in annealing temperature per cycle), extension at 72 °C for 30 secs, then another 25 cycles of denaturation at 94 °C for 30 secs, annealing at 53 °C for 30 secs, extension at 72 °C for 30 secs and a final extension step at 72 °C for 10 min. All the amplified products were run on 2% agarose gel (formed in 1× TAE buffer and ethidium bromide stain). The agarose gel electrophoresis was performed at 110 volts for 30 min. The results were visualized on gel documentation system under UV light (Voytas, 2000).

### *2.5 The restriction fragment length polymorphism assay*

The developed direct PCR was followed by an RFLP assay (Meyer et al., 1995) to discriminate species in meat samples without the need of sequencing the amplified products. This reduced the time and cost of confirmation of meat origin substantially. *Tas*I restriction endonuclease was used to generate species-specific fragments. Restriction digestion reaction mixture was prepared by mixing 10 µL PCR product, 2 µL buffer B (10×), 0.5 µL *Tas*I enzyme (5 U) and 7.5 µL of water in the PCR tube to maintain the final volume of 20 µL. The mixture was short spun and tubes were placed in an incubator for 2 hrs at 65 °C. The restricted products were then confirmed by running on 3% agarose gel.

### *2.6 Sensitivity test*

The sensitivity test was performed to estimate the minimum volume of DNA template that could yield detectable amplicons using each selected buffer and the developed direct PCR approach. So, cow and buffalo meat was subjected to DNA extraction by alkaline lysis buffers 1, 2 and 3 at 10 min boiling duration. The amount of meat and the overall volume of the buffer were 10 mg and 150 µL, respectively. Amplification was performed by taking 0.25 µL, 0.5 µL, 1 µL and 2 µL of the supernatant from each extracted sample as DNA template in 25 µL of reaction mixture and subjected to direct PCR. All reactions were carried out in duplicates along with positive and negative control reactions.

### *2.7 Specificity test*

Specificity test was conducted to assess the extraction capability of each buffer for meat samples from different species. The test also analyzed the capacity of the established direct PCR assay for specific amplification of the targeted region of the eight different species that are mostly consumed (buffalo, cow, goat, sheep and chicken) or likely to be adulterated with (horse, camel and dog). DNA was extracted from meat tissues by boiling them for 10 min in alkaline buffers 1, 2 and 3. The amount of meat and the overall volume of the buffer were 10 mg and 150 µL, respectively. Amplification was performed by taking 0.5 µL of the template DNA in 25 µL of reaction mixture. All PCR reactions were carried out in duplicates along with positive and negative control reactions.

### *2.8 Repeatability test*

The repeatability test was conducted to assess the capability of each buffer to provide positive results repeatedly under the same conditions. The test was also applied to ascertain the robustness and applicability of the developed direct PCR protocol. Total forty different commercial mutton and beef samples (10 cooked and 10 uncooked beef; 10 cooked and 10 uncooked mutton samples) were collected from the local markets and restaurants of Lahore (Punjab, Pakistan). Crude DNA was extracted from all samples by boiling them in alkaline lysis buffers 1, 2 and 3 for 10 min. The amount of meat and the overall volume of the buffer were 10 mg and 150 µL, respectively. PCR amplifications were performed by taking 0.5 µL of the template DNA in 25 µL of reaction mixture and subjected to direct PCR according to the conditions mentioned above. All PCR reactions were carried out in duplicates along with positive and negative control reactions.

### *2.9 Application for commercial food products*

A total of 53 different types of commercially prepared meat products (cooked and uncooked mutton and beef food items)

were collected from the local markets and restaurants of Lahore (Punjab, Pakistan) and stored at -20 °C until needed for DNA extraction. These samples were tested by the developed direct PCR-RFLP workflow to assess its applicability and effectiveness in such circumstances. Crude DNA was extracted from meat samples by boiling them in alkaline lysis buffer 3 for 10 min. The amount of meat and the volume of the buffer were 10 mg and 150 µL, respectively. PCR amplifications were performed by taking 0.5 µL of the template DNA in 25 µL of reaction mixture. Direct PCR and RFLP were performed according to the conditions mentioned above. All PCR reactions were carried out in duplicates along with positive and negative control reactions.

## 3 Results

### *3.1 Comparison of five boiling DNA preparation methods*

The initial comparison to assess overall DNA yield of the boiling methods revealed that PBS and distilled water produced zero results (See Figure 1A). For that reason, further experiments for comparison and optimization of suitable buffer were carried out with alkaline lysis buffers 1, 2 and 3 only (See Figure 1B). In order to evaluate their efficiency, the sample weight (10 mg, 25 mg, 50 mg), buffer volume (150 µL, 200 µL, 500 µL) and boiling durations (10 min, 20 min, 30 min) were varied. The experiments led to the conclusion that 10 mg tissue sample in 150 µL buffer volume, boiled for 10 min duration, provides sufficient amount of crude DNA while being economical, less laborious, and time-efficient, simultaneously.

### *3.2 Restriction fragment length polymorphism assay*

The direct PCR-RFLP assay was carried out for the identification of eight species i.e. buffalo, cow, sheep, goat, chicken, camel, horse and dog. All species were clearly distinguishable after running the restricted products on a 3% agarose gel, by forming species-specific patterns (See Figure 2). Table 2 provides details of specific banding patterns of all eight species understudy.

### *3.3 Specificity test*

Eight species including buffalo, cow, goat, sheep, horse, camel, chicken and dog were targeted for evaluating the specificity of the developed direct PCR approach. Detectable PCR products were obtained for each of the eight species. The most consistent results were obtained with direct PCR using alkaline

**Table 2.** Species-specific DNA banding pattern of the PCR-amplified 16S rRNA gene region restricted by *Tas*I restriction endonuclease.

| Lane | Species | Banding pattern in base pairs | | | | |
|---|---|---|---|---|---|---|
| **1** | Cow | 319 | 49 | -- | -- | -- |
| **2** | Buffalo | 190 | 180 | -- | -- | -- |
| **3** | Camel | 276 | 65 | 26 | -- | -- |
| **4** | Chicken | 179 | 162 | 26 | -- | -- |
| **5** | Dog | 181 | 72 | 50 | 49 | 26 |
| **6** | Horse | 163 | 113 | 72 | -- | -- |
| **7** | Sheep | 319 | 49 | -- | -- | -- |
| **8** | Goat | 202 | 118 | 49 | -- | -- |

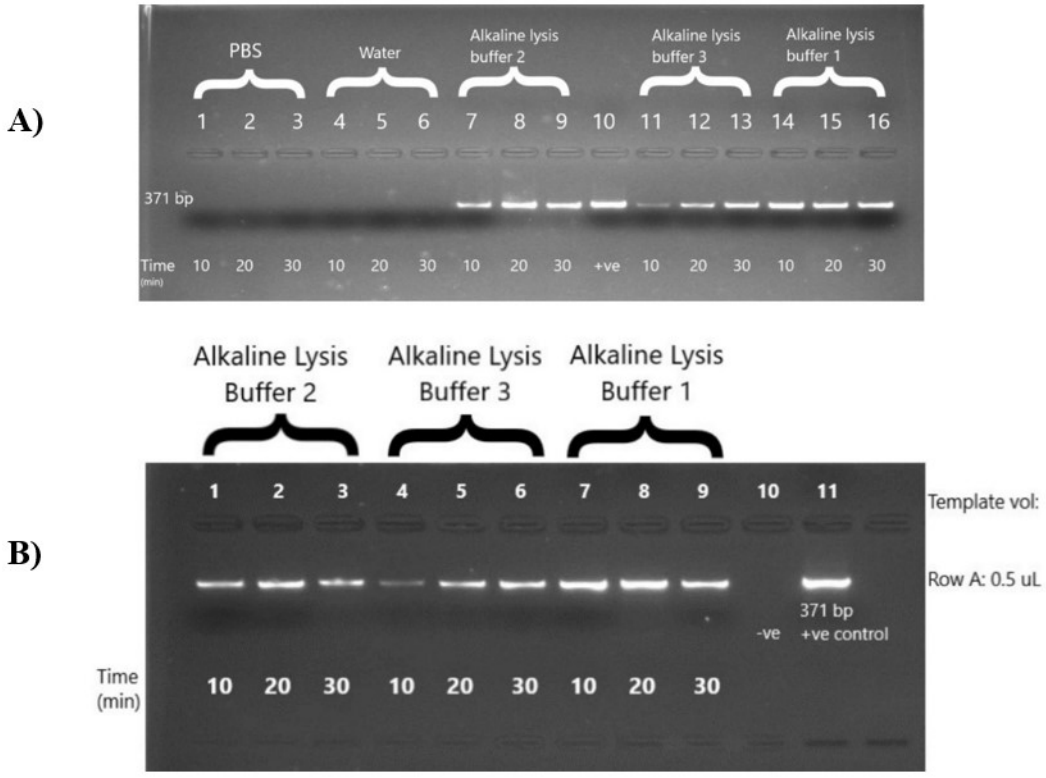


**Figure 1.** Comparison of PCR amplification of DNA prepared by different boiling methods. A): Amplification of DNA prepared by five different boiling DNA extraction methods and three different boiling times (10 min, 20 min and 30 min). B): Comparison of amplification for selected boiling DNA preparation methods using 50 mg sample and 500 µL buffer for three different boiling times (10 min, 20 min, 30 min).

lysis buffer 3. The only exception observed was chicken meat. Amplification results with chicken meat were not consistently positive. The reason might be that chicken meat has less DNA to be extracted than its organs (Buntjer et al., 1999; Ballin et al., 2009). Direct PCR amplification of crude DNA extracted by alkaline lysis buffer 1 and 2 was unsuccessful to produce any results for horse meat sample even after repeated experiments with the same conditions.

### *3.4 Sensitivity test*

For sensitivity test varying amounts of DNA template (prepared using the three selected buffers) were taken for direct PCR. The minimum amount of DNA template with which detectable PCR products were obtained was 0.25 µL, but the results were not consistently positive with this amount. However, it was observed that PCR amplifications with 0.5 µL DNA template gave consistently positive results and thus was determined as the optimum amount of DNA template for the developed direct PCR-RFLP workflow.

### *3.5 Repeatability test*

The standardized assay was validated on a total of 40 beef and mutton (raw and cooked) samples for its repeatability, robustness and applicability (as shown in Table 3). It was observed that highest amplification success rates were achieved with alkaline lysis buffer 3, while alkaline lysis buffer 1 and 2 were slightly less efficient in providing positive amplifications. This could either be due to the presence of PCR inhibitors or due to the possible variability of each alkaline lysis buffer to lyse different types of meat tissues and/or cells.

### *3.6 Application for confirmation of meat origin*

Fifty-three commercial food samples were tested including cooked and uncooked meat samples, the details of which are provided in Table S1. All samples were successfully amplified and restricted. The samples were assigned to their relevant species according to the species-specific banding patterns given in Table 2. The details of each sample and the results for RFLP are described in Table S1. The most promising results were obtained for samples boiled in alkaline lysis buffer 3. Three out of 53 samples were found to be adulterated with undeclared meat species. The falsifications were detected only in processed meat products, containing minced or shredded mutton. No horse and/or dog species were detected in commercial samples. Figure 3A-3B show the restriction patterns of different cooked and uncooked beef and mutton samples.

## 4 Discussion

The efficiency of species identification assays mostly relies on two main components; first, the extracted genomic DNA and second, the selected genomic region for identification. With time several methods have been developed either by following different PCR approaches (Song et al., 2018; Skouridou et al., 2019; Mokhtar et al., 2020; Batule et al., 2020; Yan et al., 2022) or by varying the genomic regions (Marchetti et al., 2020; Suryawan et al., 2020; Li et al., 2021; Tao et al., 2022) or both. Among such attempts, the direct PCR approach allows PCR

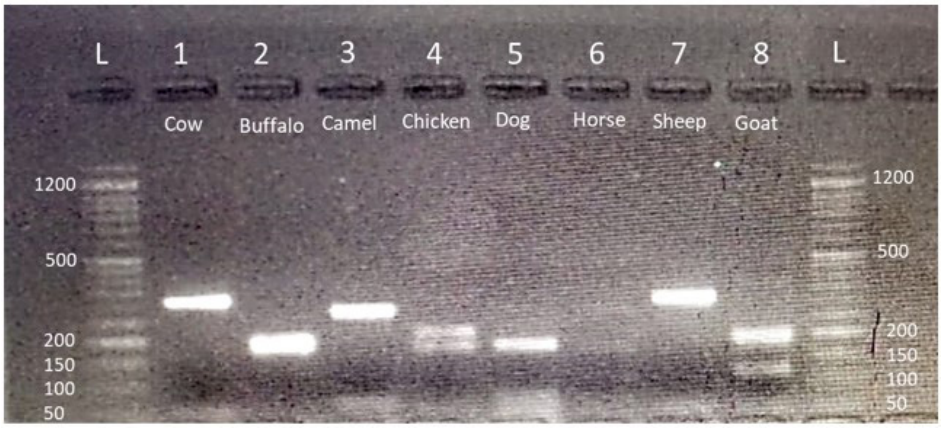


**Figure 2.** Restriction fragment length polymorphism for cow, buffalo, camel, chicken, dog, horse, sheep and goat.

**Table 3.** Amplification success rates for repeatability test of the developed direct PCR-RFLP workflow in different samples.

| S# | Type of sample | Sample size | Cooked/ Uncooked | Amplification success rate | | |
|---|---|---|---|---|---|---|
| | | | | Alkaline lysis buffer 1 | Alkaline lysis buffer 2 | Alkaline lysis buffer 3 |
| **1.** | Beef samples | 10 | Uncooked | 100% | 80% | 100% |
| **1.** | | 10 | Cooked | 90% | 100% | 90% |
| **2.** | Mutton samples | 10 | Uncooked | 90% | 100% | 100% |
| **1.** | | 10 | Cooked | 100% | 90% | 100% |

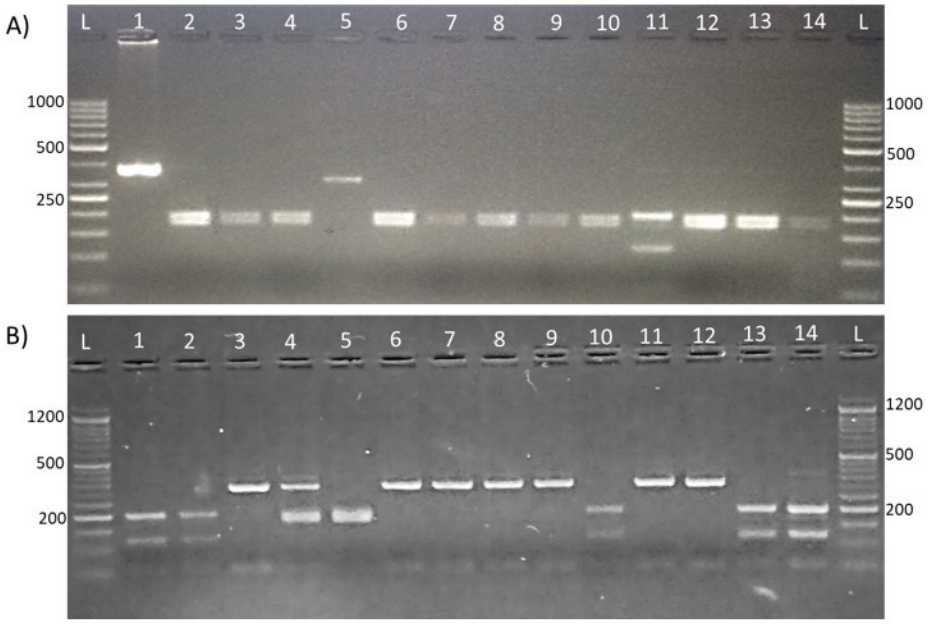


**Figure 3.** Application of the developed direct PCR-RFLP assay. A): Samples of uncooked and cooked beef. B): Samples of cooked and uncooked mutton. **L** is for Ladder. (Details for each lane are described in Table S1).

amplification without prior DNA extraction. This method efficiently reduces the time and cost of a developed assay for confirmation of meat origin (Guan et al., 2019). The said method has been successfully exploited for medical diagnostics, forensic purposes, meat origin identification, and DNA barcoding of insects, microbial and fungal fauna as well as for certain invertebrates (Kitpipit et al., 2014a; Tjhie et al., 1994; Thanakiatkrai et al., 2019; Werblow et al., 2016; Wu et al., 2020).

The quality, quantity and purity of extracted genomic DNA play a critical role in its further molecular processing (Martincová & Aghová, 2020). The most commonly used conventional DNA extraction with Phenol/Chloroform/Isoamyl alcohol (PCI) (Sambrook & Russell, 2001) requires harmful reagents and a lot of additional steps such as multiple centrifugations, which are laborious and time-consuming. On the other hand, commercial kits although provide fine quality of genomic DNA, are expensive and often replaceable by easier methods for meat identification (Mounika et al., 2021). Therefore, we first compared five different boiling DNA extraction methods and then optimized the most efficient boiling method to extract DNA from 10 mg meat tissue samples by boiling them in 150 µL lysis buffer for 10 min. This crude DNA extraction was coupled with a newly developed direct PCR-RFLP workflow for simple, time-saving and cost-effective analysis of meat origin in food products. The direct PCR-RFLP workflow was more economical and rapid as compared to Sanger sequencing. Moreover, meat samples of eight species (buffalo, cow, goat, sheep, camel, horse, chicken and dog) were successfully discriminated by this workflow with high specificity, sensitivity, and repeatability.

Commercially available meat and meat products are most susceptible to adulteration, being difficult to tease apart if mixing of two or more species under a single label is done (Kane & Hellberg, 2016; Fengou et al., 2021). Comparably, cooked food items that contain meat are even more difficult to authenticate as extensive heat and mixing complicates the identifying process to the next level (Perestam et al., 2017; Xing et al., 2020). Therefore, we also successfully analyzed fifty-three commercially cooked and uncooked, mutton and beef samples (from local markets and restaurants of Lahore (Punjab, Pakistan)) for the validation of the developed direct PCR-RFLP assay.

The boiling DNA preparation methods have been previously applied to shorten the labor intensive DNA extraction protocols, reducing the time to 10-30 min (Labrador et al., 2019; Girish et al., 2020; Narushima et al., 2020; Mounika et al., 2021; Girish et al., 2022). The developed procedure has several special aspects that make it desirable for species identification especially for confirmation of meat origin. First, the lengthy 2-3 days DNA extraction protocol is shortened to only 10 min of boiling the sample in a buffer and then directly proceeding it to PCR, hence the term 'direct PCR' (Guan et al., 2019). Second, as no purification steps are included in the extraction procedure through boiling methods, therefore inhibitors can hamper the PCR reaction (Schrader et al., 2012). However, if very small volume about 0.25 µL to 2 µL of template DNA is subjected to a 25 µL PCR reaction, the concentrations of inhibitors will be lowered to such values that they may not hinder the amplification (Tagliavia et al., 2016).

Although, all three buffers yielded promising results and can be used for further RFLP analysis, in this study only alkaline lysis buffer 3 was selected for developing the direct PCR-RFLP assay. The selection was made on several criteria: first, the positive PCR amplifications were slightly higher with alkaline lysis buffer 3 than the other two buffers. Second, AL 1 and 2 were unable to give results for horse DNA sample unlike AL 3, which isolates the DNA from meat tissue of horse as well as identifies horse and dog species accurately in halal meat products because these

are majorly mistrusted as non-halal adulterants (Nagpal, 2008; Yamoah & Yawson, 2014). On the other hand, PCR amplifications of uncooked chicken meat DNA were not consistently positive with AL 3 and can be considered a major drawback of this buffer. As horse meat has also been part of food adulteration scandal and a lot of public mistrust in the past (Premanandh, 2013), it was preferred that the developed assay be capable to analyze horse meat DNA. Our buffer selection coincides with most of the recent studies that have adapted alkaline lysis for DNA extraction (Koch et al., 2019; Martincová & Aghová, 2020; Bui et al., 2021) as they have used AL 3 as their lysis buffer. The developed fast extraction protocol of DNA might be helpful for quicker PCR-based identification of meat species in testing laboratories.

Another desirable aspect of the developed direct PCR-RFLP assay is the newly designed 16S rRNA primers. These primers have the potential to encompass the whole vertebral fauna allowing species discrimination with specificity and sensitivity. Mitochondrial DNA as compared to the nuclear DNA is preferred for distinguishing meat species owing to the fact that there are approximately (1000-10,000) copies of this organelle in a single cell and its high genetic variability among different species (Ballin et al., 2009; Chen et al., 2010; Kowalczyk et al., 2021). Moreover, alkaline method of DNA extraction is preferred for the mitochondrial DNA separation over the nuclear one due to the low stability of nuclear DNA after abrupt change in pH during extraction procedure (Borgo et al., 1996). 16S rRNA holds high inter-species DNA variations and low intra-species DNA variation (Taniguchi et al., 2022), providing high confidence in meat species discrimination. Within the mitochondrial genes, 16S rRNA has been used for broad range of mammalian and birds' species because of its evolutionary stability (Vences et al., 2016; Ha et al., 2017; Lalitha & Chandavar, 2017).

Furthermore, the developed direct PCR method is coupled with RFLP assay which is much more desirable for molecular based meat identification, especially, in cases where large number of samples have to be processed as it does not require DNA sequencing and/or specialized equipment and reduces the cost and time for post-PCR processing of samples (Guan et al., 2019; Gargouri et al., 2021; Vaithiyanathan et al., 2021; Taha et al., 2021). It has been mostly applied for species discrimination in processed and unprocessed meat products because of its simplicity, quicker detection and reduced cost (Al et al., 2020; Asghar et al., 2022; Farag et al., 2022).

## 5 Conclusion

In conclusion, this study shows that meat identification by direct PCR-RFLP assay is rapid, specific, sensitive, and repeatable. Efficient and cost-effective DNA extraction can be achieved with alkaline lysis method by processing only 10 mg of meat sample and boiling it in 150 µL buffer for 10 min. The comparison of different boiling DNA preparation methods for a direct PCR approach led to the conclusion that out of the five buffers under study, all three alkaline lysis buffers can be utilized for direct PCR-RFLP assay but alkaline lysis buffer 3 (25 mM NaOH, 0.2 mM $Na_2EDTA$) is preferable on the basis of positive amplification rate and capability to extract crude DNA from all the targeted species. The direct PCR-RFLP assay developed in this study can provide a simpler and affordable meat authentication test for laboratories as well as authorities dealing with food adulteration.

## Conflict of interest

The authors declare that they have no personal or financial competing interest.

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## References

Al, S., Hizlisoy, H., Onmaz, N. E., Karadal, F., Güngör, C., Yildirim, Y., & Gönülalan, Z. (2020). The determination of meat species by PCR-RFLP method using mitochondrial ND4 gene in pastırma, a traditional dry cured meat product. *Turkish Journal of Veterinary and Animal Sciences*, 44(1), 35-41. http://dx.doi.org/10.3906/vet-1907-116.

Alasaad, S., Sánchez, A., García-Mudarra, J. L., Jowers, M. J., Pérez, J. M., Marchal, J. A., Romero, I., Garrido-García, J. A., & Soriguer, R. C. (2012). Single-tube HotSHOT technique for the collection, preservation and PCR-ready DNA preparation of faecal samples: the threatened Cabrera's vole as a model. *European Journal of Wildlife Research*, 58(1), 345-350. http://dx.doi.org/10.1007/s10344-011-0526-x.

Ali, N., Rampazzo, R. D. C. P., Costa, A. D. T., & Krieger, M. A. (2017). Current nucleic acid extraction methods and their implications to point-of-care diagnostics. *BioMed Research International*, 2017, 9306564. http://dx.doi.org/10.1155/2017/9306564. PMid:28785592.

Asghar, U., Malik, M. F., Rashid, U., Ashraf, N. M., Afsheen, S., & Hashim, M. (2022). Identification of meat species by PCR-RFLP method using single set of degenerative primers. *Sarhad Journal of Agriculture*, 38(1), 188-194. http://dx.doi.org/10.17582/journal.sja/2022/38.1.188.194.

Ballin, N. Z., Vogensen, F. K., & Karlsson, A. H. (2009). Species determination–can we detect and quantify meat adulteration? *Meat Science*, 83(2), 165-174. http://dx.doi.org/10.1016/j.meatsci.2009.06.003. PMid:20416768.

Batule, B. S., Seok, Y., & Kim, M. G. (2020). An innovative paper-based device for DNA extraction from processed meat products. *Food Chemistry*, 321, 126708. http://dx.doi.org/10.1016/j.foodchem.2020.126708. PMid:32251924.

Ben-Amar, A., Oueslati, S., & Mliki, A. (2017). Universal direct PCR amplification system: a time- and cost-effective tool for high-throughput applications. *3 Biotech*, 7(4), 246. http://dx.doi.org/10.1007/s13205-017-0890-7. PMid:28711981.

Besbes, N., Sáiz-Abajo, M. J., & Sadok, S. (2022). Comparative study of DNA extraction to initiate harmonized protocol for a simple method of species identification: fresh and canned tuna case study. *CYTA: Journal of Food*, 20(1), 39-49. http://dx.doi.org/10.1080/19476337.2021.2020337.

Borgo, R., Souty-Grosset, C., Bouchon, D., & Gomot, L. (1996). PCR-RFLP analysis of mitochondrial DNA for identification of snail meat species. *Journal of Food Science*, 61(1), 1-4. http://dx.doi.org/10.1111/j.1365-2621.1996.tb14712.x.

Bui, S., Dalvin, S., Vågseth, T., Oppedal, F., Fossøy, F., Brandsegg, H., Jacobsen, Á., Norði, G., Fordyce, M. J., Michelsen, H. K., Finstad, B., & Skern-Mauritzen, R. (2021). Finding the needle in the haystack: comparison of methods for salmon louse enumeration in plankton

samples. *Aquaculture Research*, 52(8), 3591-3604. http://dx.doi.org/10.1111/are.15202.

Buntjer, J. B., Lamine, A., Haagsma, N., & Lenstra, J. A. (1999). Species identification by oligonucleotide hybridisation: the influence of processing of meat products. *Journal of the Science of Food and Agriculture*, 79(1), 53-57. http://dx.doi.org/10.1002/(SICI)1097-0010(199901)79:1<53::AID-JSFA171>3.0.CO;2-E.

Cai, Z., Zhong, G., Liu, Q., Yang, X., Zhang, X., Zhou, S., Zeng, X., Wu, Z., & Pan, D. (2022). Molecular authentication of twelve meat species through a promising two-tube hexaplex polymerase chain reaction technique. *Frontiers in Nutrition*, 9, 813962. http://dx.doi.org/10.3389/fnut.2022.813962. PMid:35399682.

Chen, S. Y., Liu, Y. P., & Yao, Y. G. (2010). Species authentication of commercial beef jerky based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene. *Journal of Genetics and Genomics*, 37(11), 763-769. http://dx.doi.org/10.1016/S1673-8527(09)60093-X. PMid:21115170.

Farag, W. M. H., Aljuaydi, S. H., Galal, M. K., Kassem, G. M. A., & Gouda, E. M. (2022). Efficient mitochondrial genes in the characterization of meat species ap-plying PCR-RFLP technique. *Advances in Animal and Veterinary Sciences*, 10(1), 8-13. http://dx.doi.org/10.17582/journal.aavs/2022/10.1.8.13.

Fengou, L. C., Tsakanikas, P., & Nychas, G. J. E. (2021). Rapid detection of minced pork and chicken adulteration in fresh, stored and cooked ground meat. *Food Control*, 125, 108002. http://dx.doi.org/10.1016/j.foodcont.2021.108002.

Gargouri, H., Moalla, N., & Kacem, H. H. (2021). PCR–RFLP and species-specific PCR efficiency for the identification of adulteries in meat and meat products. *European Food Research and Technology*, 247(9), 2183-2192. http://dx.doi.org/10.1007/s00217-021-03778-y.

Girish, P. S., Barbuddhe, S. B., Kumari, A., Rawool, D. B., Karabasanavar, N. S., Muthukumar, M., & Vaithiyanathan, S. (2020). Rapid detection of pork using alkaline lysis-Loop Mediated Isothermal Amplification (AL-LAMP) technique. *Food Control*, 110, 107015. http://dx.doi.org/10.1016/j.foodcont.2019.107015.

Girish, P. S., Haunshi, S., Vaithiyanathan, S., Rajitha, R., & Ramakrishna, C. (2013). A rapid method for authentication of buffalo (Bubalus bubalis) meat by alkaline lysis method of DNA extraction and species specific polymerase chain reaction. *Journal of Food Science and Technology*, 50(1), 141-146. http://dx.doi.org/10.1007/s13197-011-0230-6. PMid:24425899.

Girish, P. S., Kumari, A., Gireesh-Babu, P., Karabasanavar, N. S., Raja, B., Ramakrishna, C., & Barbuddhe, S. B. (2022). Alkaline lysis-loop mediated isothermal amplification assay for rapid and on-site authentication of buffalo (Bubalus bubalis) meat. *Journal of Food Safety*, 42(1), e12955. http://dx.doi.org/10.1111/jfs.12955.

Guan, F., Jin, Y., Zhao, J., Ai, J., & Luo, Y. (2019). A novel direct PCR lysis buffer can improve PCR from meat matrices. *Food Analytical Methods*, 12(1), 100-107. http://dx.doi.org/10.1007/s12161-018-1342-7.

Ha, J., Kim, S., Lee, J., Lee, S., Lee, H., Choi, Y., Oh, H., & Yoon, Y. (2017). Identification of pork adulteration in processed meat products using the developed mitochondrial DNA-based primers. *Korean Journal for Food Science of Animal Resources*, 37(3), 464-468. http://dx.doi.org/10.5851/kosfa.2017.37.3.464. PMid:28747833.

Haunshi, S., Pattanayak, A., Bandyopadhaya, S., Saxena, S. C., & Bujarbaruah, K. M. (2008). A simple and quick DNA extraction procedure for rapid diagnosis of sex of chicken and chicken embryos. *Journal of Poultry Science*, 45(1), 75-81. http://dx.doi.org/10.2141/jpsa.45.75.

Iqbal, M., Saleem, M. S., Imran, M., Khan, W. A., Ashraf, K., Zahoor, M. Y., Rashid, I., Rehman, H. U., Nadeem, A., Ali, S., Naz, S., & Shehzad, W. (2020). Single tube multiplex PCR assay for the identification of banned meat species. *Food Additives & Contaminants. Part B*, 13(4), 284-291. http://dx.doi.org/10.1080/19393210.2020.1778098. PMid:32552602.

Kane, D. E., & Hellberg, R. S. (2016). Identification of species in ground meat products sold on the US commercial market using DNA-based methods. *Food Control*, 59, 158-163. http://dx.doi.org/10.1016/j.foodcont.2015.05.020.

Kang, T. S., & Tanaka, T. (2018). Comparison of quantitative methods based on SYBR Green real-time qPCR to estimate pork meat adulteration in processed beef products. *Food Chemistry*, 269, 549-558. http://dx.doi.org/10.1016/j.foodchem.2018.06.141. PMid:30100472.

Kibbe, W. A. (2007). OligoCal: an online oligonucleotide properties calculator. *Nucleic Acids Research*, 35(Suppl. 2), W43-W46. http://dx.doi.org/10.1093/nar/gkm234.

Kieleczawa, J. (2006). *DNA sequencing II: optimizing preparation and cleanup* (Vol. 2). Sudbury: Jones & Bartlett Learning.

Kitpipit, T., Chotigeat, W., Linacre, A., & Thanakiatkrai, P. (2014a). Forensic animal DNA analysis using economical two-step direct PCR. *Forensic Science, Medicine, and Pathology*, 10(1), 29-38. http://dx.doi.org/10.1007/s12024-013-9521-8. PMid:24435950.

Kitpipit, T., Sittichan, K., & Thanakiatkrai, P. (2014b). Direct-multiplex PCR assay for meat species identification in food products. *Food Chemistry*, 163, 77-82. http://dx.doi.org/10.1016/j.foodchem.2014.04.062. PMid:24912698.

Koch, H. R., Blohm-Sievers, E., & Liedvogel, M. (2019). Rapid sex determination of a wild passerine species using loop-mediated isothermal amplification (LAMP). *Ecology and Evolution*, 9(10), 5849-5858. http://dx.doi.org/10.1002/ece3.5168. PMid:31161003.

Kowalczyk, M., Staniszewski, A., Kamińska, K., Domaradzki, P., & Horecka, B. (2021). Advantages, possibilities, and limitations of mitochondrial DNA analysis in molecular identification. *Folia Biologica*, 69(3), 101-111.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547-1549. http://dx.doi.org/10.1093/molbev/msy096. PMid:29722887.

Labrador, K., Agmata, A., Palermo, J. D., Follante, J., & Pante, M. J. (2019). Authentication of processed Philippine sardine products using Hotshot DNA extraction and minibarcode amplification. *Food Control*, 98, 150-155. http://dx.doi.org/10.1016/j.foodcont.2018.11.027.

Lalitha, R., & Chandavar, V. R. (2017). Species identification inferred through mitochondrial 16s rrna gene sequence between freshwater testudines-lissemys punctata and melanochelys trijuga. *International Journal of Advanced Research*, 5(7), 2231-2239. http://dx.doi.org/10.21474/IJAR01/4964.

Li, J., Li, J., Liu, R., Wei, Y., & Wang, S. (2021). Identification of eleven meat species in foodstuff by a hexaplex real-time PCR with melting curve analysis. *Food Control*, 121, 107599. http://dx.doi.org/10.1016/j.foodcont.2020.107599.

Linacre, A., Pekarek, V., Swaran, Y. C., & Tobe, S. S. (2010). Generation of DNA profiles from fabrics without DNA extraction. *Forensic Science International. Genetics*, 4(2), 137-141. http://dx.doi.org/10.1016/j.fsigen.2009.07.006. PMid:20129473.

Mansouri, M., Khalilzadeh, B., Barzegari, A., Shoeibi, S., Isildak, S., Bargahi, N., Omidi, Y., Dastmalchi, S., & Rashidi, M. R. (2020). Design a highly specific sequence for electrochemical evaluation of meat adulteration in cooked sausages. *Biosensors & Bioelectronics*, 150, 111916. http://dx.doi.org/10.1016/j.bios.2019.111916. PMid:31818752.

Marchetti, P., Mottola, A., Piredda, R., Ciccarese, G., & Pinto, A. (2020). Determining the authenticity of shark meat products by DNA sequencing. *Foods*, 9(9), 1194. http://dx.doi.org/10.3390/foods9091194. PMid:32872285.

Martincová, I., & Aghová, T. (2020). Comparison of 12 DNA extraction kits for vertebrate samples. *Animal Biodiversity and Conservation*, 43(1), 67-77. http://dx.doi.org/10.32800/abc.2020.43.0067.

Meyer, R., Höfelein, C., Lüthy, J., & Candrian, U. (1995). Polymerase chain reaction–restriction fragment length polymorphism analysis: a simple method for species identification in food. *Journal of AOAC International*, 78(6), 1542-1551. http://dx.doi.org/10.1093/jaoac/78.6.1542. PMid:8664595.

Mokhtar, N. F. K., Sheikha, A. F., Azmi, N. I., & Mustafa, S. (2020). Potential authentication of various meat-based products using simple and efficient DNA extraction method. *Journal of the Science of Food and Agriculture*, 100(4), 1687-1693. http://dx.doi.org/10.1002/jsfa.10183. PMid:31803942.

Mounika, T., Girish, P. S., Kumar, M. S., Kumari, A., Singh, S., & Karabasanavar, N. S. (2021). Identification of sheep (Ovis aries) meat by alkaline lysis-loop mediated isothermal amplification technique targeting mitochondrial D-loop region. *Journal of Food Science and Technology*, 58(10), 3825-3834. http://dx.doi.org/10.1007/s13197-020-04843-2. PMid:34471306.

Murugaiah, C., Noor, Z. M., Mastakim, M., Bilung, L. M., Selamat, J., & Radu, S. (2009). Meat species identification and Halal authentication analysis using mitochondrial DNA. *Meat Science*, 83(1), 57-61. http://dx.doi.org/10.1016/j.meatsci.2009.03.015. PMid:20416658.

Nagpal, S. (2008). *Asia-Pacific News. Foreign workers eating dog meat in Malaysia as food prices rise.* Retrieved from https://topnews.in/foreign-workers-eating-dog-meat-malaysia-food-prices-rise-249306

Narushima, J., Kimata, S., Soga, K., Sugano, Y., Kishine, M., Takabatake, R., Mano, J., Kitta, K., Kanamaru, S., Shirakawa, N., Kondo, K., & Nakamura, K. (2020). Rapid DNA template preparation directly from a rice sample without purification for loop-mediated isothermal amplification (LAMP) of rice genes. *Bioscience, Biotechnology, and Biochemistry*, 84(4), 670-677. http://dx.doi.org/10.1080/09168451.2019.1701406. PMid:31842715.

Njaramba, J. K., Wambua, L., Mukiama, T., Amugune, N. O., & Villinger, J. (2021). Species substitution in the meat value chain by high-resolution melt analysis of mitochondrial PCR products. bioRxiv. In press. https://doi.org/10.1101/2021.01.12.426171.

Perestam, A. T., Fujisaki, K. K., Nava, O., & Hellberg, R. S. (2017). Comparison of real-time PCR and ELISA-based methods for the detection of beef and pork in processed meat products. *Food Control*, 71, 346-352. http://dx.doi.org/10.1016/j.foodcont.2016.07.017.

Premanandh, J. (2013). Horse meat scandal–a wake-up call for regulatory authorities. *Food Control*, 34(2), 568-569. http://dx.doi.org/10.1016/j.foodcont.2013.05.033.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, 39(21), e145. http://dx.doi.org/10.1093/nar/gkr732. PMid:21930509.

Sajali, N., Wong, S. C., Hanapi, U. K., Jamaluddin, S. A. B., Tasrip, N. A., & Desa, M. N. M. (2018). The challenges of DNA extraction in different assorted food matrices: a review. *Journal of Food Science*, 83(10), 2409-2414. http://dx.doi.org/10.1111/1750-3841.14338. PMid:30184265.

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schnepf, N., Scieux, C., Resche-Riggon, M., Feghoul, L., Xhaard, A., Gallien, S., Molina, J. M., Socie, G., Viglietti, D., Simon, F., Mazeron, M. C., & Legoff, J. (2013). Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. *Journal of Clinical Microbiology*, 51(7), 2096-2102. http://dx.doi.org/10.1128/JCM.00067-13. PMid:23616450.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors–occurrence, properties and removal. *Journal of Applied Microbiology*, 113(5), 1014-1026. http://dx.doi.org/10.1111/j.1365-2672.2012.05384.x. PMid:22747964.

Sepp, R., Szabo, I., Uda, H., & Sakamoto, H. (1994). Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *Journal of Clinical Pathology*, 47(4), 318-323. http://dx.doi.org/10.1136/jcp.47.4.318. PMid:8027368.

Sheikha, A. F. (2019). DNAFoil: novel technology for the rapid detection of food adulteration. *Trends in Food Science & Technology*, 86, 544-552. http://dx.doi.org/10.1016/j.tifs.2018.11.012.

Sheikha, A. F., Mokhtar, N. F. K., Amie, C., Lamasudin, D. U., Isa, N. M., & Mustafa, S. (2017). Authentication technologies using DNA-based approaches for meats and halal meats determination. *Food Biotechnology*, 31(4), 281-315. http://dx.doi.org/10.1080/08905436.2017.1369886.

Skouridou, V., Tomaso, H., Rau, J., Bashammakh, A. S., El-Shahawi, M. S., Alyoubi, A. O., & O'Sullivan, C. K. (2019). Duplex PCR-ELONA for the detection of pork adulteration in meat products. *Food Chemistry*, 287, 354-362. http://dx.doi.org/10.1016/j.foodchem.2019.02.095. PMid:30857710.

Song, K. Y., Hwang, H. J., & Kim, J. H. (2018). Data for the optimization of conditions for meat species identification using ultra-fast multiplex direct-convection PCR. *Data in Brief*, 16, 15-18. http://dx.doi.org/10.1016/j.dib.2017.11.004. PMid:29167814.

Suryawan, G. Y., Suardana, I. W., & Wandia, I. N. (2020). Sensitivity of polymerase chain reaction in the detection of rat meat adulteration of beef meatballs in Indonesia. *Veterinary World*, 13(5), 905-908. http://dx.doi.org/10.14202/vetworld.2020.905-908. PMid:32636586.

Swaran, Y. C., & Welch, L. (2012). A comparison between direct PCR and extraction to generate DNA profiles from samples retrieved from various substrates. *Forensic Science International. Genetics*, 6(3), 407-412. http://dx.doi.org/10.1016/j.fsigen.2011.08.007. PMid:21925992.

Tagliavia, M., Nicosia, A., Salamone, M., Biondo, G., Bennici, C. D., Mazzola, S., & Cuttitta, A. (2016). Development of a fast DNA extraction method for sea food and marine species identification. *Food Chemistry*, 203, 375-378. http://dx.doi.org/10.1016/j.foodchem.2016.02.095. PMid:26948627.

Taha, K. M., Khdr, D. M., & Kareem, K. Y. (2021). Identification and differentiation of poultry meat and products using PCR-RFLP technique. *Mesopotamia Journal of Agriculture*, 49(1), 34-42. http://dx.doi.org/10.33899/magrj.2021.129251.1105.

Taniguchi, K., Akutsu, T., Watanabe, K., Ogawa, Y., & Imaizumi, K. (2022). A vertebrate-specific qPCR assay as an endogenous internal control for robust species identification. *Forensic Science International. Genetics*, 56, 102628. http://dx.doi.org/10.1016/j.fsigen.2021.102628. PMid:34798377.

Tao, D., Xiao, X., Lan, X., Xu, B., Wang, Y., Khazalwa, E. M., Pan, W., Ruan, J., Jiang, Y., Liu, X., Li, C., Ye, R., Li, X., Xu, J., Zhao, S., & Xie, S. (2022). An inexpensive CRISPR-based point-of-care test for the identification of meat species and meat products. *Genes*, 13(5), 912. http://dx.doi.org/10.3390/genes13050912. PMid:35627297.

Thanakiatkrai, P., Dechnakarin, J., Ngasaman, R., & Kitpipit, T. (2019). Direct pentaplex PCR assay: an adjunct panel for meat species

identification in Asian food products. *Food Chemistry*, 271, 767-772. http://dx.doi.org/10.1016/j.foodchem.2018.07.143. PMid:30236743.

Tjhie, J. H., Van Kuppeveld, F. J., Roosendaal, R., Melchers, W. J., Gordijn, R., MacLaren, D. M., Walboomers, J. M., Meijer, C. J., & van den Brule, A. J. (1994). Direct PCR enables detection of mycoplasma pneumoniae in patients with respiratory tract infections. *Journal of Clinical Microbiology*, 32(1), 11-16. http://dx.doi.org/10.1128/jcm.32.1.11-16.1994. PMid:7510308.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *BioTechniques*, 29(1), 52-54. http://dx.doi.org/10.2144/00291bm09. PMid:10907076.

Vaithiyanathan, S., Vishnuraj, M. R., Reddy, G. N., & Srinivas, C. (2021). Authentication of camel meat using species-specific PCR and PCR-RFLP. *Journal of Food Science and Technology*, 58(10), 3882-3889. http://dx.doi.org/10.1007/s13197-020-04849-w. PMid:34471312.

Vences, M., Lyra, M. L., Perl, R. G., Bletz, M. C., Stanković, D., Lopes, C. M., Jarek, M., Bhuju, S., Geffers, R., Haddad, C. F. B., & Steinfartz, S. (2016). Freshwater vertebrate metabarcoding on Illumina platforms using double-indexed primers of the mitochondrial 16S rRNA gene. *Conservation Genetics Resources*, 8(3), 323-327. http://dx.doi.org/10.1007/s12686-016-0550-y.

Voytas, D. (2000). Agarose gel electrophoresis. *Current Protocols in Molecular Biology*, 51(1), 2-5. https://doi.org/10.1002/0471142727.mb0205as51.

Werblow, A., Flechl, E., Klimpel, S., Zittra, C., Lebl, K., Kieser, K., Laciny, A., Silbermayr, K., Melaun, C., & Fuehrer, H. P. (2016). Direct PCR of indigenous and invasive mosquito species: a time-and cost-effective technique of mosquito barcoding. *Medical and Veterinary Entomology*, 30(1), 8-13. http://dx.doi.org/10.1111/mve.12154. PMid:26663040.

Wu, H., Qian, C., Wang, R., Wu, C., Wang, Z., Wang, L., Zhang, M., Ye, Z., Zhang, F., He, J., & Wu, J. (2020). Identification of pork in raw meat or cooked meatballs within 20 min using rapid PCR coupled with visual detection. *Food Control*, 109, 106905. http://dx.doi.org/10.1016/j.foodcont.2019.106905.

Xing, R. R., Hu, R. R., Han, J. X., Deng, T. T., & Chen, Y. (2020). DNA barcoding and mini-barcoding in authenticating processed animal-derived food: a case study involving the Chinese market. *Food Chemistry*, 309, 125653. http://dx.doi.org/10.1016/j.foodchem.2019.125653. PMid:31670116.

Yamoah, F. A., & Yawson, D. E. (2014). Assessing supermarket food shopper reaction to horsemeat scandal in the UK. *International Review of Management and Marketing*, 4(2), 98-107. Retrieved from https://dergipark.org.tr/en/pub/irmm/issue/32080/355050

Yan, S., Lan, H., Wu, Z., Sun, Y., Tu, M., & Pan, D. (2022). Cleavable molecular beacon-based loop-mediated isothermal amplification assay for the detection of adulterated chicken in meat. *Analytical and Bioanalytical Chemistry*, 414(28), 8081-8091. http://dx.doi.org/10.1007/s00216-022-04342-7. PMid:36152037.

Yu, N., Ren, J., Huang, W., Xing, R., Deng, T., & Chen, Y. (2021). An effective analytical droplet digital PCR approach for identification and quantification of fur-bearing animal meat in raw and processed food. *Food Chemistry*, 355, 129525. http://dx.doi.org/10.1016/j.foodchem.2021.129525. PMid:33799266.

Yue, G. H., & Orban, L. (2001). Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. *Marine Biotechnology*, 3(3), 199-204. http://dx.doi.org/10.1007/s10126-001-0010-9. PMid:14961356.

Zhao, G., Shen, X., Liu, Y., Xie, P., Yao, C., Li, X., Sun, Y., Lei, Y., & Lei, H. (2021). Direct lysis-multiplex polymerase chain reaction assay for beef fraud substitution with chicken, pork and duck. *Food Control*, 129, 108252. http://dx.doi.org/10.1016/j.foodcont.2021.108252.

Zieritz, A., Yasaeng, P., Razak, N. F. A., Hongtrakul, V., Kovitvadhi, U., & Kanchanaketu, T. (2018). Development and evaluation of hotshot protocols for cost-and time-effective extraction of PCR-ready DNA from single freshwater mussel larvae (Bivalvia: Unionida). *The Journal of Molluscan Studies*, 84(2), 198-201. http://dx.doi.org/10.1093/mollus/eyy011.

## Supplementary Material

Supplementary material accompanies this paper.

**Table S1**. Application of the developed direct PCR-RFLP assay on 53 different commercial meat products.

This material is available as part of the online article from https://doi.org/10.1590/fst.65122