Application of a multiplex polymerase chain reaction (mPCR) assay to detect fraud by substitution of bovine meat cuts with water buffalo meat in Northern Brazil

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Abstract

The adulteration of meat products can affect the confidence of consumers and the market, leading to negative impacts on the economy. Accordingly, product authenticity has become an important issue in modern society. Therefore, our study aimed to optimize the extraction of DNA from meat and use multiplex polymerase chain reaction (mPCR) to determine the incidence of fraud by substitution of bovine meat cuts with water buffalo meat in the states of Pará and Amapá, Northern Brazil. The mPCR protocol used primers that amplify sequences of 346 base pairs of bovine DNA and 220 base pairs of buffalo DNA. To assess the sensitivity of the technique, a standardized PCR assay was performed using the template DNA extracted and diluted from $10^{-1}$ to $10^{-10}$ in PCR-grade water. Next, 161 samples of meat cuts marketed as bovine (rump) origin were collected in the states of Pará and Amapá, Northern Brazil. The mPCR assays demonstrated good specificity of the primers used. The sensitivity test amplified bovine and buffalo DNA fragments down to the $10^{-2}$ dilution. The results demonstrated fraud by substitution of beef by water buffalo meat in 21.7% of samples, demonstrating that this act does occur intentionally for economic gains.

1. Introduction

Food fraud is prevalent worldwide, and recent studies have focused on identifying fraud in meat products (Ahmed et al., 2018; Mousavi et al., 2015; Veneza, da Silva, Sampaio, Schneider, & Gomes, 2017). In Brazil, the adulteration of food products, mainly meat products, has affected consumer confidence and the international market, leading to negative impacts on the Brazilian economy (National Hog Farmer, 2017). Consequently, product authenticity has become an important issue in modern society (Premanandh, 2013; Shehata et al., 2017).

Brazil is a major player in cattle breeding, possessing the largest commercial herd in the world, and Brazilian beef is exported to several countries (Gomes, Feijó, & Chiari, 2017; IBGE, 2017). The variety of soil, climate, and ecosystems makes cattle breeding considerably heterogeneous in the country, being able to meet the demands of different national or international markets (Oaigen et al., 2011). Northern Brazil is an important livestock region, being responsible for 22.6% of cattle production. Furthermore, this region is also important for breeding buffaloes, owning 66% of the national herd, with the states of Pará and Amapá leading the country (IBGE, 2017). However, the water buffalo production chain in this region is relatively unorganized and...
lacks coordination strategies, with buffalo meat being under-valued and cheaper than cattle meat (Marques et al., 2015).

The low value of buffalo meat is due to lack of adequate legislation for the production of water buffaloes in the country. Accordingly, animals are slaughtered and sold like cattle in many places (Teixeira, Teixeira, Caldeira, Bastianetto, & Oliveira, 2012). Similarities in characteristics such as color, aroma, flavor, and texture between beef and buffalo meat, besides the identical commercial cuts between these animals, makes it difficult for consumers to identify the difference between the products at the time of purchase (Joele et al., 2017; Lira et al., 2005). Moreover, although Brazil has a tracking system (SISBOV), it has many limitations in its application (IBGE, 2017; Rodrigues & Nantes, 2010). All these factors contribute to the occurrence of commercial fraud by substitution of beef meat with water buffalo meat (Marques et al., 2016).

Food fraud is a threat to health and consumer confidence, in addition to generating economic losses to businesses, with loss of market share (Ballin, 2010; Van Ruth, Luning, Silvis, Yang, & Huisman, 2018). Fraud in the meat industry and retail markets occurs mainly by replacing the meat of one species with another undeclared species that is usually cheaper, in order to obtain higher profits (Kyrova et al., 2017).

One of the techniques being studied and recommended for identifying food fraud is polymerase chain reaction (PCR), which is prominent owing to its rapidity and efficiency. This method is considered to be very promising because it is highly sensitive, as minimal amounts of DNA can be detected using species-specific primers (Egito et al., 2006).

Several studies have demonstrated the efficiency of PCR in detecting adulteration in meat products (Amaral, Santos, Oliveira, & Mafra, 2017; Oliveira et al., 2018; Quinto, Tinoco, & Hellberg, 2016; Xu et al., 2018), and some studies using multiplex PCR (mPCR) have already been developed for detecting bovine and buffalo species in different products, such as milk (Darwish, Allam, & Amin, 2009), cheese (López-Calleja et al., 2005), minced meat (Oliveira et al., 2015), and raw and processed products (Wang, Hang, & Geng, 2018). However, additional research involving detection of fraud by substitutions in commercially available in natura meat cuts are needed, particularly in predominantly agricultural countries such as Brazil, which supply meat to various markets. Furthermore, the majority of scientific studies involving fraud are related to meat byproducts. Thus, fraud that is detected in animal byproducts may be directly related to fraud by substitution of meat cuts.

Given the important issues related to consumer protection, health, and future implications of Brazilian beef in local and international trade, the present study aimed to optimize a meat DNA extraction methodology and apply a molecular protocol based on mPCR to verify the occurrence of commercial fraud by the replacement of meat cuts labelled and presented as beef with water buffalo meat in the states of Pará and Amapá, Northern Brazil.

2. Material and methods

2.1. Materials

DNA from the species Bos taurus and Bubalus bubalis, obtained from samples of bovine and buffalo meat, respectively, were collected and labelled in a slaughterhouse registered by the Federal Inspection Service (SIF) of Brazil.

2.2. DNA extraction

DNA extraction was performed using the Wizard® Promega extraction kit, according to the manufacturer’s instructions, with modifications. Accordingly, 0.3 g of each meat sample was weighed in a vial, then 800 μL of lysis solution and 10 μL of protease K were added. The samples were incubated in a water bath at 56°C overnight (14 h) and centrifuged at 12,000 × g for 10 min. To optimize the separation and precipitation of protein, 700 μL of phenol-chloroform (1:1) was added, incubated on ice for 5 min, and an additional centrifugation was performed at 14,000 × g for 20 min. Four hundred microliters of the supernatant was transferred to a new vial containing 600 μL of isopropanol, which was then centrifuged at 14,000 × g for 1 min. The supernatant was completely discarded, and 600 μL of 70% ethanol was added and centrifuged at 14,000 × g for 1 min. The supernatant was again discarded and the vials were dried in an oven at 37°C for 30 min. DNA samples were hydrated with 100 μL TE buffer solution (10 mM Tris-HCl, 1 mM EDTA) and stored at −18°C until use. Additionally, a reagent blank was included with each set of extracted samples as negative control.

2.3. DNA quality and concentration

The quality of the extracted DNA was analyzed by 1% agarose gel electrophoresis in TBE buffer (Tris-Borate-EDTA 0.5X), stained with non-mutagenic GelRed™ fluorescent dye, run in TBE buffer 0.5X at 110V and visualized in a transilluminator under ultraviolet light coupled to Image Lab™ Software (Biorad). The nucleic acid concentration was determined using a NanoDrop® spectrophotometer.

2.4. Specific primers and mPCR for gene amplification

The mPCR methodology was performed as described by Darwish et al. (2009), with modifications described by Oliveira et al. (2015). For this purpose, primers were used to amplify specific sequences of the species B. bubalis 125BUF-REV2 (TTCTATTTTCTTGTGTGTTGGGTT), B. taurus 125BT-REV2 (AATAGGGTTATGCACCTGAACTCAT), and primers that amplify common sequences to the two species 12SM-FW (CTAGAGGACGCTGTTCTATACTGATA) described by López-Calleja et al. (2005), which amplify fragments of 346 bp for B. taurus and 220 bp for B. bubalis. The primers were prepared according to the manufacturer’s instructions (Ludwing Biotec®), and eluted in TE buffer pH 8.0 to a concentration of 100 pmol μL⁻¹.

The mPCR mix was run in a final volume of 25 μL containing: 2.5 μL of 10X buffer solution (100 mM Tris-HCl and 500 mM KCl), 0.75 μL of MgCl₂ (50 mM), 0.5 μL of dNTP (10 mM mix), 1.0 μL containing 5 pmol of each primer, 3.0 μL of bovine and buffalo DNA template at a concentration of 100 ng μL⁻¹ each, and 0.5 μL of Taq DNA polymerase (1 U μL⁻¹). Ultra-pure water (11.75 μL) was added to reach the final volume. A negative control of the reaction was performed by replacing DNA with ultra-pure water.

The reactions were performed in a thermocycler (Applied Biosystems VERITI™ 96) programmed for an initial denaturation at 93°C for 3 min, then 30 cycles of denaturation at 93°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, plus a final extension at 72°C for 10 min.
2.5. Electrophoresis of mPCR products

The amplified fragments were homogenized in 1 µL of GelRed™ dye and electrophoresed in a 1.5% agarose gel, run in 0.5X TBE buffer at 110 V. Results were analyzed with the aid of a photodocumentation equipment under ultraviolet light associated with Image Lab™ Software (Biorad).

2.6. Specificity and sensitivity

The specificity of the test was assessed by applying the reaction to four other species: swine (Sus scrofa domesticus), sheep (Ovis aries), domestic fowl (Gallus domesticus), and salmon (Salmo salar), purchased and certified directly in slaughterhouses supervised by the SIF in Brazil. The DNA of the samples was extracted according to the protocol described above.

The sensitivity of the technique was evaluated by serially diluting the DNA of target species in triplicate from an initial concentration of 223 ng µL⁻¹ (beef) and 231 ng µL⁻¹ (buffalo) diluted up to a concentration of 10⁻¹⁰ in Tris-EDTA buffer. These dilutions were tested by the proposed methodology to determine the minimum concentration that can be detected by the technique.

2.7. Application of the mPCR in samples of commercial meat

A total of 161 commercial samples of meat cuts labelled as cattle from different suppliers were collected from butchers (n = 51), free-trade stalls (n = 57), supermarkets (n = 14), and meat stalls (n = 38). The samples were collected in the state of Amapá (Macapá) and in the state of Pará (Belém, Marabá, Marajó, and Castanhal), which together account for the largest buffalo production in Brazil and the largest bovine production in the northern region of the country, according to the Brazilian Institute of Geography and Statistics (IBGE) (IBGE, 2017). Due to the scarcity of published data, the sample number was calculated taking into account the district number of each locality, considering an estimated prevalence of fraud ranging from 1–50% determined according to the method proposed by Barbetta (2002) and Spiegel, Schiller, and Srinivasan (2004) for a 95% confidence interval and a tolerable sampling error of 5%, with a total of 49 samples from Belém, 38 from Marabá, 32 from Marajó, 28 from Castanhal, and 14 from Macapá.

The samples were collected randomly in their original packaging, simulating actual purchasing conditions by the consumer. They were then stored in a refrigerated container and forwarded to the laboratory of Hygiene and Food Quality of the Institute of Veterinary Medicine of the Federal University of Pará – Campus Castanhal, where their origin was registered and the sample was then stored at −18°C until analysis.

2.8. Electrophoresis of the mPCR products of the commercial samples

The extraction of DNA and the mPCR reaction were carried out as described above. For each electrophoresis run, a negative control and a bovine and a buffalo positive control of amplicons obtained from mPCR were used, which were homogenized in 1 µL of GelRed™ dye and subjected to 1.5% agarose gel electrophoresis, run in 0.5X TBE buffer. Results were analyzed with the aid of a transilluminator under ultraviolet light coupled to Image Lab™ Software (Biorad).

3. Results and discussion

3.1. DNA extraction and quality

The optimization of the extraction technique ensured genomic DNA with a mean concentration of 223 ± 3.6 ng µL⁻¹ (bovine) and 231 ± 2.6 ng µL⁻¹ (buffalo). According to Marengoni, Machado, and Gasparino (2006), studies that optimize the extraction of good quality DNA are important in the field of molecular biology. Scorsato and Telles (2011) assert that isolating nucleic acids from tissues with sufficient quantity, purity, and integrity is essential for ensuring that the desired regions are amplified. Extraction of DNA using proteinase K in the digestion buffer and phenol-chloroform in the purification step resulted in material with satisfactory quantity and purity for the amplification reaction.

3.2. mPCR

The results demonstrated that the protocol adopted enabled the simultaneous amplification of several genes. Although the primers have already been used by other authors for identifying fraud in milk, cheese, and meats (López-Calleja et al., 2005; Oliveira et al., 2018; Silva et al., 2015), this is the first time they have been used for identifying fraud in commercially available whole meat cuts, which suggests that they may also be used to detect B. taurus and B. bubalis DNA in other food products.

3.3. Specificity and sensitivity

The specificity test showed that our mPCR protocol did not amplify DNA from the species S. scrofa domesticus, O. aries, G. domesticus, and S. salar from samples analyzed in this study. According to Ali et al. (2015), this is related to the specific binding ability of the set of primers to the target sequences under study. Rashid et al. (2015) stated that mPCR may have reduced efficiency or even fail at amplification because of the inability of the primer to anneal to their respective binding regions. Oliveira et al. (2018) used the same primers to verify the authenticity of minced meat and found no cross-reaction in the specificity test.

The specificity of the mPCR demonstrated that this technique was able to simultaneously amplify DNA from cattle (B. taurus) and buffalo (B. bubalis) up to a dilution of 10⁻² (2.23 ng µL⁻¹ and 2.31 ng µL⁻¹, respectively). The results show that this range is adequate to identify the DNA of these meat species using the mPCR conditions proposed in this study. A similar result was found by Oliveira et al. (2015) for buffalo meat using the same primers, in which the detection limit was 2.15 ng µL⁻¹. However, the outcome for beef was superior, with a sensitivity of 0.041 ng µL⁻¹.

Although the sensitivity of the proposed mPCR was adequate, better results were obtained by other authors working with different meat species, such as Hou et al. (2015) who reported a sensitivity of 0.05 ng µL⁻¹ for DNA from chicken, ducks, and geese, Fang and Zhang (2016) who...
observed a detection limit of 0.1 ng μL⁻¹ for murine DNA (mouse or rat) in meat products, and Qin, Hong, Kim (2016) who detected a limit of 0.005 ng μL⁻¹ for beef (B. taurus), duck (Anas platyrhynchos), and lamb (O. aries). Nevertheless, Oliveira et al. (2015) reported that the variation in sensitivity is a natural phenomenon of mPCR depending on the species involved, target gene, and fragment size. Thus, although specificity and sensitivity tests have already been performed in other studies, we decided to perform them again in our study since we used a different DNA extraction methodology and different concentrations of mPCR reagents.

### 3.4. mPCR in commercial meat samples

The results obtained from the mPCR performed in samples of meat cuts sold commercially in the North region of Brazil (Pará and Amapá) are shown in Figure 1.

Approximately 21.7% (35/161) of the samples labelled and presented as bovine meat cuts were actually water buffalo meat cuts, indicating fraud by substitution. Of these samples, 18% (29/35) were from the state of Pará and 3.7% (6/35) from the state of Amapá. Table 1 shows the distribution of the results of the samples analyzed.

![Figure 1](image.png)

**Figure 1.** Agarose gel (1.5%) stained with GelRed™, demonstrating fraud by substitution of bovine meat cuts with water buffalo meat in the states of Pará and Amapá, Northern Brazil. A: L: 1-kb molecular marker; Bt: B. taurus – bovine positive control (346 bp); Bb: Bubalus bubalis – positive buffalo control (220 bp); B1 to B5: fraud in Belém (PA); M6 and M7: fraud in Marabá (PA); J8 to J12: fraud in the Marajó (PA); C-: Negative control. B: L: 1-kb molecular marker; Bt: B. taurus – bovine positive control (346 bp); Bb: B. bubalis – positive buffalo control (220 bp); J13 to J24: fraud in the Marajó (PA); C: Negative control. C: L: 1-kb molecular marker; Bt: B. taurus – bovine positive control (346 bp); Bb: B. bubalis – positive buffalo control (220 bp); J25 to J29: continued fraud in the Marajó (PA); A30 to A35: fraud in Macapá (AP); C-: Negative control.

### Table 1. Distribution of the results of the mPCR analysis used to detect fraud by replacement of bovine meat cuts with buffalo meat in samples of meat cuts collected from different locations in the states of Pará and Amapá, Northern Brazil.

| Locations      | DNA samples positive for beef (%) | DNA samples positive for buffalo (%) | Total samples (%) |
|---------------|----------------------------------|-------------------------------------|-------------------|
| Belém (PA)    | 24 (15)                          | 6 (3.7)                             | 30 (18.6)         |
| Marabá (PA)   | 36 (22.4)                        | 2 (1.2)                             | 38 (23.6)         |
| Marajó (PA)   | 10 (6.2)                         | 22 (13.7)                           | 32 (19.9)         |
| Castanhal (PA)| 28 (17.4)                        | 0 (0.0)                             | 28 (17.4)         |
| Macapá (AP)   | 8 (5.0)                          | 6 (3.7)                             | 14 (8.7)          |
| Total         | 126 (78.3)                       | 35 (21.7)                           | 161 (100.0)       |

In a study performed to detect different species in meat and meat byproducts, Ayaz, Ayaz, and Erol (2006) found that 22.2% of samples of raw meat declared as bovine were actually horse meat and/or venison. Similarly, Kane and Hellberg (2016), examined the authenticity of meat sold on the commercial market in the United States also found that, of the 48 samples analyzed, 10 were substituted with other species of meat.

The results obtained corroborate data obtained by Oliveira et al. (2018) and Silva et al. (2015), demonstrating that adulteration of food products with undeclared species is widespread in Brazil, indicating economically motivated fraud. Hossain et al. (2017) reported that the motivation for adulteration stems from a company’s interest in obtaining a growing profit from improper sale of similar but cheaper items. According to Fang and Zhang (2016), besides distorting market rules, this practice threatens food security and can affect people’s health.

Of the locations with a higher incidence of fraud by substituting beef meat cuts with water buffalo meat, Marajó (PA) and Macapá (AP) lead the ranking in the studied region, probably because these regions possess a larger number of buffaloes than other localities. Unlike other places like Egypt, Vietnam, and the European Union, in which buffalo meat is valued, in Brazil, especially in the northern region, this material presents a low commercial value, being 20% cheaper than beef, according to Marques et al. (2016). Hossain et al. (2017) found similar results when analyzing the authenticity of meat products in Malaysia, and identified the total replacement of beef by buffalo, as this is cheaper.

In the North Region of Brazil, the wide availability of water buffaloes and the lack of specific regulation for the slaughter and marketing of buffalo meat mean that these animals are usually slaughtered clandestinely or in slaughterhouses that do not fulfill minimum requirements of sanitary legislation, contributing to fraudulent actions. According to Brasil (1990), illegal slaughter is considered a crime in the country for undermining consumer relations. In addition, Omotosho, Emikpe, Lasisi, and Oladunjoye (2016) report that this type of practice directly influences the quality of meat and has implications for public health, since there may be a risk of transmitting zoonoses, such as listeriosis, staphylococcal infections, and salmonellosis, among others.

The fragility of the buffalo production chain in most regions of Brazil contributes to these animals being registered, slaughtered, and marketed as beef. Food fraud is considered a crime in Brazil according to the Penal Code (Brazil, 1940), because it undermines public health. According to Santos et al. (2016) one
way to contain these fraudulent actions would be to organize the sector, standardization, and valuation of water buffalo meat in Brazil, which is currently a significant challenge.

Regarding the percentage of fraud found by establishment type, 54% (19/35) were obtained from butchers, followed by 40% (14/35) from free-trade markets, and 6% (2/35) from supermarkets. According to Santos, Moura, and Camara (2011) and Martins and Ferreira (2018), butchers and free-trade markets are the locations most prone to fraud, given the ease in obtaining products without verifying the origin and the lack of effective inspection by regulatory bodies in these establishments. To change this, the authorities need to invest in surveillance and implement control measures to reduce the risks of this vulnerability. These measures include improving the program for verifying the origin of meat available in the market and offering tools that make it possible to verify the authenticity of the products marketed, such as the application of molecular techniques.

The data found in this study are concerning, since the replacement of beef meat cuts with water buffalo meat in the Brazilian market can directly affect the lifestyle of individuals and can generate a serious problem to consumers who are allergic to other types of meat as described by Ali et al. (2012) and Sentandreu and Sentandreu (2014). Therefore, correct information about the authenticity of foods contained on labels is essential to ensure consumer safety. Thus, molecular techniques, such as PCR and its variations, are being established to generate rapid and reliable results for authenticating meat species in food products.

4. Conclusion

The results of this study demonstrate that the fraud by substitution of beef meat cuts with water buffalo meat occurs frequently and intentionally in the northern region of Brazil to increase economic gains, despite the fact that Brazilian legislation does not permit the counterfeiting of food products with undeclared species.

The results of this study are significant since the northern region of Brazil is a potential producer of meat products for both domestic and international markets, requiring more effective surveillance in order to contain these acts of fraud throughout the country and to ensure consumer protection and the credibility of the country in the meat export market.

The mPCR protocol applied in this study was able to detect the species B. taurus and B. bubalis in whole meat cuts without nonspecific pairings of the primers, and may serve as an alternative for the routine surveillance of these products.

Acknowledgments

We gratefully acknowledge the help and assistance provided by the Dean of Research and Graduate Studies of the Federal University of the State of Pará (PROPESP) and the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil).

Declaration of interest

The authors declare that have no conflict of interest that might constitute an embarrassment to the publication of this article submitted to CyTA-Journal of Food.

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