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EVALUATION OF A MULTIPLEX PCR FOR SIMULTANEOUS DETECTION OF *Bos taurus*, *Bubalus bubalis* AND *Salmonella* spp. IN GROUND BEEF MARKETED IN MARAJÓ ISLAND

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ABSTRACT

The present work intends to use a mPCR for simultaneous detection of *Bos taurus*, *Bubalus bubalis* and *Salmonella* spp. in samples commercially available on Marajó Island, Pará State, Brazil. For this purpose, a mPCR reaction with different concentrations of DNA and primers with different annealing temperatures was optimized. Next, 28 samples of ground meat marketed as being of bovine origin were collected and a mPCR for the determination of species and for the detection of *Salmonella* spp. were realized. The results demonstrated the efficiency of the proposed technique for identification of *Bos taurus*, *Bubalus bubalis* and *Salmonella* spp. We conclude that the proposed mPCR technique is efficient and that buffalo meat is marketed as bovine on the Island of Marajó and that such frauds violating the current legislation that normalizes the commercialization of ground beef.

Keywords: *Bubalus bubalis*, buffaloes, species identification, adulteration, fraud, bovine

INTRODUCTION

Most places in the world have a strong cultural identity, related to historical architecture, collections in museums, handicrafts, and some are distinguished by the cuisine, characterized by the use of ingredients or sources of animal protein of species almost exclusively of a region (Molinillo and Japutra, 2016). An outstand place is the Island of Marajó, in the Brazilian Amazon, a group of historical islands with lots of architectural aspects, like its cuisine and food products from the buffaloes, such as milk, meat and its derivatives (Seixas *et al*., 2014).

In the last registry of the Brazilian Institute of Geography and Statistics, the brazilian herd of buffalo was 1,261,922 heads, with 64% concentrated in the northern region of the country, and the state of Pará owned 36% of the national flock and the largest concentration of buffalo was

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distributed in the municipalities of the Marajó Island (IBGE, 2012). The indices show the great productive and commercial impact that the buffalo may represent in the meat market in the North region and in Brazil as a whole.

Among the products of animal origin marketed, the ground beef stands out, considering it is a food that is consumed by the general population, due to its more affordable price, practicality and preparing recipes. (Cabrera and Saadoun, 2014).

Throughout the manipulation process involved in its production of ground meat, and taking into account the area/volume ratio, this food may present a greater fragility to the incidence of fraudulent practices, the probable substitution or addition of similar meats, besides greater possibility of problems related to microbiological contamination, in particular the risks associated with *Salmonella* spp. (Sousa *et al*., 2012).

Recent epidemiological data indicate that *Salmonella* spp. was the agent most commonly found in outbreaks resulting of food poisoning (Cheung and Kam, 2012). And according to the Resolution of the Board of Directors (RDC Nº 12 of 2001) the fresh meats are fit for consumption only when there is absence of this bacterial genus for each 25 g of sample (BRASIL, 2001). In this way, a tool that simultaneously determines the presence of fraud and *Salmonella* spp., in meat products is of paramount importance.

Up to this moment, several authors have demonstrated that the Polymerase Chain Reaction (PCR) can be an important tool for the detection of fraud in ground meat or meat products (Oliveira *et al*., 2015; Kitpipit *et al*., 2014; Chen *et al*., 2010), as well as for the detection of *Salmonella* spp. (Soumet *et al*., 1999), but the multiplex PCR research of the species *Bubalus bubalis*, *Bos taurus* and of this bacterial genus has not yet been reported.

The objective of this work was to evaluate the efficiency of a multiplex PCR for simultaneous detection of the species *Bos taurus*, *Bubalus bubalis* and the bacterial genus *Salmonella* spp., in order to verify the incidence of fraud and contamination in samples of ground meat commercialized in Marajó Island, state of Pará, Brazil.

MATERIALS AND METHODS

For the accomplishment of the present research, initially strain of *Salmonella tiphymurium* (American Type Culture Collection 14028) was used as positive control. Additionally, bovine and buffalo ground meat samples were collected directly from a refrigerated slaughterhouse certified by the brazilian federal inspections services and were used in the standardization as a positive control for the techniques being analyzed in this study.

Afterwards, samples of ground meat commercialized as being of bovine origin, available in commercial establishments in the municipalities of Soure, Salvaterra, Joanes and Camará, located in the Marajó Island, were collected and transported in their original containers to the Food Hygiene and Quality Laboratory, School of Veterinary Medicine, Federal University of Pará - Castanhal, for the accomplishment of the planned analyzes.

The amount of samples collected was calculated based on the marketing points in each municipality, according to previous research carried out by the scientific team and given the lack of data in the literature, considering an estimated adulteration prevalence ranging from 1 to 50% and determined according to the method proposed by Spiegel *et al*. (2004); Barbetta *et a*l. (2004), for a 95% confidence interval and a 5% tolerable

sampling error.

Next, the DNA of all samples (the bovine and buffalo meat used as positive control of the commercial samples as well as *Salmonella tiphymurium*) was extracted using the protocol described by Darwish *et al*. (2009), but with modifications as suggested by Silva *et al*. (2015). The samples were separated using 0.8% agarose gel (Inlab™) electrophoresis (Bio-Rad), run in Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer, which contains Tris-Base (Promega), boric acid (Alphatec) and 0.5% EDTA (Ludwig), and stained with 6X Safer dye (KASVI) (1 µL of the dye/5 µL of the sample). The analysis of the electrophoresis results was performed using a UV transilluminator (Gel Documentation System, Gel Doc™, Bio-Rad). DNA quantification and purity determination was performed using a BioTek Gen5™ spectrophotometer at 230 nm, 260 nm and 280 nm according to the Beer-Lambert law (Lambert, 1760; Beer, 1852).

The multiplex PCR and qPCR reactions were performed with primers amplifying specific sequences for the buffalo species (primer reverse buf 5'-TTCATAATAACTTTCGTGTTGTTGGGTGT - 3'), the bovine species (primer reverse bov 5'-AAATAGGGTTAGATGCACTGAATCCAT - 3'), and sequences common to both species (primer forward buf/bov 5' - CTAGAGGAGCCTGTTCTATAATCGATA - 3'), which were previously described by López-Calleja et al. (2005), and specific to Salmonella spp. (primer reverse 5'-ACTGGTAAAGATGGCT-3' and primer forward 5' - CGGTGTTGCCCAGGTTGGTAAT - 3'), described by Soumet *et al*. (1999). These primers generate sequences of 220 bp for Bubalus bubalis DNA, 346 bp for *Bos taurus* DNA and 429 bp for *Salmonella* spp., the primers were prepared according to the manufacturer's instructions (Ludwing Biotec) and were eluted in TE buffer (pH 8.0) to a concentration of 100 pmol/µL.

In the search for the best efficiency in the amplification, 4 treatments were tested for each annealing temperature programmed (52ºC, 53ºC, 54ºC, 55ºC and 56ºC), with different concentrations of DNA (1 and 2 µL of 241 ng/µL of control DNA) and two distinct concentrations of primers (5 and 10 pmol).

The proposed multiplex PCR methodology was applied according to methods previously described by Darwish *et al*. (2009), with some modifications as suggested by Oliveira *et al*. (2015). The PCR solution contained 50 mM KCl (Ludwing Biotec), 10 mM Tris-HCl (Ludwing Biotec), 10X buffer, 10 mM dNTP mix (Ludwing Biotec), approximately 241 ng of each template DNA, 1U Taq DNA Polymerase (Ludwing Biotec), 5 pmol of bovine and buffalo primer and 10 pmol of Salmonella spp. primer (Ludwing Biotec) and sterilized ultrapure water to a final volume of 25 µL per reaction. The thermocycler (Applied Biosystems VERITI 96) was programmed for 35 cycles, with denaturing, annealing and extension temperatures and times of 94°C/1 minute, 55ºC/30 seconds and 72°C/30 seconds, respectively. In addition, an initial denaturing step was performed at 93°C for 3 minutes, and a final extension step was performed at 72°C for 10 minutes. The amplicons were separated using 1.5% agarose gel (Inlab™) electrophoresis (Bio-Rad) run in TBE buffer and stained with 6X Safer dye (KASVI) (1 µL of dye/5 µL of sample). The analysis of the electrophoresis results was performed using a UV transilluminator (Gel Documentation System, Gel Doc™, Bio-Rad).

After obtaining the data, the Pearson's linear correlation was applied through BioEstat software version 5.3, aiming to determine the direct correlation between the incidence of fraud

by addition and/or replacement of bovine ground beef by buffalo and presence of contamination by *Salmonella* spp.

RESULTS

The results obtained through the Beer-Lambert law (Lambert, 1760; Beer, 1852) showed that DNA extraction resulted in an extracted material with quality and concentration sufficient for multiplex PCR and qPCR. The purity and yield of the total DNA extracted was confirmed by measuring the absorbance at 230 nm, 260 nm and 280 nm, which indicated values that met DNA purity parameters and with a mean concentration of 241 ng/µL for the *Bos Taurus*, *Bubalus bubalis* and *Salmonella* spp. samples.

The set of primers for the detection of *Bos Taurus*, *Bubalus bubalis* and *Salmonella* spp. presented good accuracy and precision in multiplex PCR. From the result of the 4 treatments it was determined that a multiplex PCR reaction with 2 µL of control DNA of the three targets (241 ng/µL), with 5 pmol of primers for *Bos taurus* and *Bubalus bubalis* and 10 pmol of primers for *Salmonella* spp., at the annealing temperature of 55°C, was the only efficient for simultaneous amplification.

The Figure 1 shows the results obtained using multiplex PCR performed on samples from the control group under ideal conditions.

A total of 28 ground meat samples sold as ground meat were collected from different stores within the target study region. Sample sizes were determined according to calculations of proposed sample numbers. The Figure 2 shows the results obtained using multiplex PCR performed in some of the samples collected in Marajó island.

Adulteration was detected in 71.4% (20/28) of the samples. Of the adulterated samples, 100% (20/20) of the samples were classified as adulteration by substitution (only buffalo DNA was detected in the samples), and 20% (4/20) of these samples were contaminated with *Salmonella* spp. In non-fraudulent samples (8/28), it was possible to detect *Salmonella* spp. in 25% (2/8).

The results obtained through the proposed statistical method showed no correlation between the incidence of fraud and contamination by *Salmonella* spp. (R = 1.0 and p = nonsignificant), thus accepting the null hypothesis.

DISCUSSION

The choice of the theme of our research is related to fraudulent practices involving bubalus bubalis species due to buffalo be the basis of the local economy and also because the population of bubalino herds is much larger than the cattle herd population in this area of study, being 7% cattle and 93% buffalo, being small producers, the largest responsible for this production. This fact was confirmed in research by Oliveira, Matos and Santana (2016).

In spite of this high superiority in the amount of buffalo herd in relation to cattle (IBGE, 2012), there is still a predominance of the commercialization of ground beef labelled as coming from the bovine species in all commercial establishments in the municipalities (Soure, Salvaterra, Joanes and Camará), in which the 28 samples analysed in the present study were collected (Adepará, 2013).

The results demonstrated here suggest that a substitution phenomenon may be occurring with bovine and buffalo meat in the Marajó Island, related to the greater buffalo herd population and

consequently greater availability of meat in relation to the low offer of the bovine species.

The detection of fraud in more than 71% of the samples of ground meat collected in the Marajó Island shows that this behaviour has been happening in the market of meat of the Island. A similar situation, involving the species *Bos taurus* and *Bubalus bubalis*, was previously reported in the northern and northeaster regions of the state of Pará and Macapá (Brazil), from the analysis of cheese samples (Silva *et al*., 2015), and in samples of ground beef in the cities of Belém, Macapá and Santarém, in northern Brazil (Oliveira *et al*., 2015).

In Marajó island, this phenomenon may be occurring because the low availability of beef has led to its fraudulent substitution for buffalo meat, that despite being a meat of high nutritional value and a regional delicacy that has considerable added value, must not be added to products labelled exclusively as bovine because this increment is considered as substitution fraud.

According to the European legislation on microbiological criteria for foodstuffs, the established standard is the analysis of multiple test portions from the same food item, of which none must be positive for a certain test portion size for *Salmonella* spp. (Ec, 2005). The European legislation on microbiological criteria for foodstuffs provides a standard for the rest of the world.

In this way, to the presence of Salmonella spp., also considering the microbiological standards for ground beef provided by Brazilian Resolution of the Board of Directors (RDC Nº 12 of 2001) it was observed that 21.4% of samples collected on the Island of Marajó not complied the Brazilian legislation, because the required standard is the absence of *Salmonella* spp. in 25 g of the product (Brasil, 2001).

However, the absence of correlation between this contamination and the incidence of fraud indicate that the high contamination in the samples of ground meat acquired in the Island of Marajó may be related to its manufacturing process, that involves microbiological risk factors related to the absence of infrastructure, standardization of products, adequate techniques of handling and reduced capacity of people, issues that were previously reported on the island related to the production of cheese (Blaskovsky *et al*., 2010).

The grinding process involved in the production of ground beef increases the area of contact of the product and presents additional steps of manipulation that raises the vulnerability of this meat derivative to fraudulent practices and the microbiological contamination, this fact was confirmed in our study by the high detection of fraud and considerable detection of Salmonella spp., in samples from the Marajó Island (Motta *et al*., 2000).

The set of primers for the identification of the species *Bos taurus* and *Bubalus bubalis*, which have been used here, has previously been used, alone or in combination, by several researchers around the world (Cantekin *et al*., 2015; Oliveira *et al*., 2015; Silva *et al*., 2015; Solmaz *et al*., 2014; Darwish *et al*, 2009; López-Calleja *et al*., 2005). In a similar way, the primers used herein for identification of the bacterial genus *Salmonella* spp. have already been used in unnippled or multiplex PCR reactions in several recent surveys (Boarini *et al*., 2015; Chagas *et al*., 2013; Moussa *et al*., 2012; Nillian *et al*., 2011; Oliveira *et al*., 2003; Soumet *et al*., 1999). However, in our research, we presented an innovative methodology of multiplex PCR, which proved efficient for simultaneous detection of *Bos taurus*, *Bubalus bubalis* and *Salmonella* spp. DNA, by the combination of the two sets of primers mentioned above.

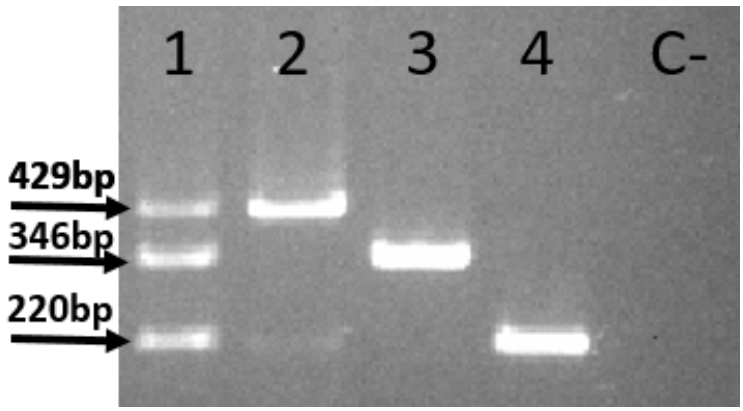

Figure 1. 1.5% agarose gel showing the presence of bovine (346 bp), buffalo (220 bp) and *Salmonella* spp. (429bp) DNA fragments obtained using multiplex PCR from the Control group under ideal conditions.

1: DNA-pool with *Salmonella* spp. control (429 bp), bovine control (346 bp) and buffalo control (220 bp); 2: *Salmonella* spp. control (429 bp); 3: bovine control (346 bp); 4: buffalo control (220 bp) and 5: negative control.

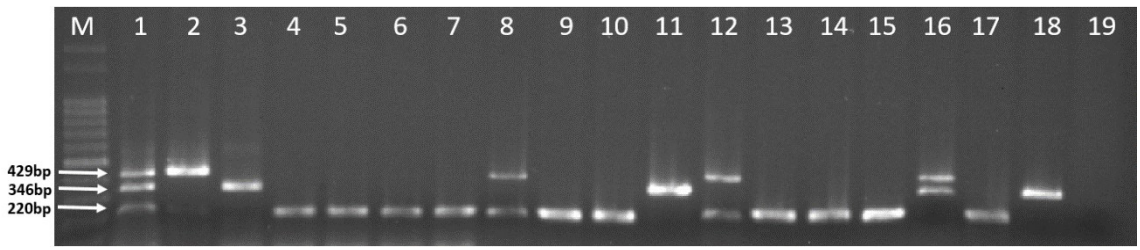

Figure 2. 1.5% agarose gel showing the presence of bovine (346 bp), buffalo (220 bp) and *Salmonella* spp. (429 bp). DNA fragments obtained using multiplex PCR performed in some of the samples colleted in Marajó island.

1: DNA-pool with *Salmonella* spp. control (429 bp), bovine control (346 bp) and buffalo control (220 bp); 2: *Salmonella* spp. control (429 bp); 3: bovine control (346 bp); 4: buffalo control (220 bp); 5 to 10, 12 to 15 and 17: adulteration by substitution with buffalo DNA (220 bp); 11, 16 and 18: absence of adulteration with cattle DNA (346 bp); 8, 12: adulteration by substitution with buffalo DNA (220 bp) and contamination with *Salmonella* spp. DNA (429 bp); 16: absence of adulteration with cattle DNA (346 bp) and contamination with *Salmonella* spp. DNA (429 bp) and 19: negative control.

The most interesting feature of the technique chosen for this work is the optimization of time and reagents by the possibility of determining all the species in question in the same PCR reaction and, because of this, many researchers have opted for similar methodologies in research involving different foods (Ali *et al*., 2015; Karabasanavar *et al*., 2013).

The development of a multiplex PCR presents some challenges, since the simultaneous detection capability in the same reaction involves the use of a set of primers that need to have their proven specificity, a single annealing temperature ideal for all the primers involved and that these do not have homologous regions so that primer dimers are not formed and thus does not compromise the success of the PCR reaction (Ali *et al*., 2015).

Due to the lack of correlation between the prevalence of fraud and the contamination with *Salmonella* spp., detected by the statistical method used here, it is demonstrated that the fraudulent practice of replacing bovine ground beef with buffalo does not affect the microbiological quality of buffalo meat consumed in Marajó Island. It is noteworthy that according to the Normative Instruction (NI) No. 83 was published by the Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura, Pecuária e Abastecimento - MAPA), of November 21, 2003 in Brazil, the ground meat can be produced from the milling of muscle masses of buffalo as long as it is specified on its respective label (Brasil, 2003). And because it is a delicacy of the cuisine Marajoara, with a proven niche market (Marques *et al*., 2015), we suggest a transparent transition in the marketing of ground beef buffalo as a substitute for ground beef bovine, without harm to those involved.

In addition, also we recommend the use of multiplex PCR, proposed here, as a tool to be used by the supervisory bodies for the authentication and quality control of ground beef marketed both in Marajó Island and in the rest of the world.

CONCLUSION

We conclude that the multiplex PCR, developed here, was efficient in the simultaneous identification of *Bos taurus* and *Bubalus bubalis* and *Salmonella* spp. and that both adulteration by substitution and contamination by *Salmonella* spp. are present in retail ground meat of the Marajó island. This fact reinforces the need for efficient methodologies for hygienic-sanitary authentication and evaluation to be established so that they can be used as a tool by the food inspection foodstuffs.

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