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Quantitative detection of pork meat by EvaGreen real-time PCR to assess the authenticity of processed meat products

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ABSTRACT

The development of highly sensitive and quantitative tools to identify undeclared pork meat is very important to authenticate processed meat products and, particularly, in the case of Halal products. Quantitative approaches are crucial to distinguish deliberate adulterations from cross-contaminations. This study intended to develop and validate a novel specific and highly sensitive Evagreen real-time PCR system for pork meat quantification in processed meat products. A normalised assay based on the ΔCt method was successfully developed and optimised, allowing the detection and quantification of levels down to 0.0001% and 0.01% (w/w) of pork meat, respectively, in both raw and thermally processed. The method was effectively validated using blind meat mixtures, exhibiting adequate parameters of trueness, precision and repeatability. Its application to several commercial samples, including Halal and regular meat products, showed the presence of undeclared pork species in 54% of the analysed samples, with 40% of Halal products presenting traces of pork, therefore not in good agreement with their label statements.

1. Introduction

Processed meat products are particularly vulnerable to adulteration, as highlighted by the recent scandals on the horsemeat in Europe and the rat meat in Asia. Such adulterations are mainly due to the substitution of high-valued meats by cheaper ones, the addition of undeclared species and the substitution of muscle protein by cheaper vegetable proteins (Ballin, 2011). Besides the economic aspects, fraud in the meat industry also raises questions regarding health issues and is considered a matter of special concern for consumers that have dietary restrictions due to ethnical options or religious practices. Among them, Muslims are of significant importance as they are estimated to represent 1.5 billion of individuals around the world and are only allowed to consume Halal meat (Nakyinsige, Man, & Sazili, 2012). In particular, the presence of undeclared pork meat and/or its derivatives, including lard, blood plasma, offal, collagen and gelatine, is a major concern since pork is strictly forbidden in Halal foods (Aida, Che Man, Wong, Raha, & Son, 2005; Nakyinsige et al., 2012). Moreover, unintentional cross-contamination and/or mislabelling can occur since several food products are currently processed in the same facilities, being sufficient to ruin the Halal status of foods in the case of pork derivatives. Therefore, it is important to determine pork ingredients in processed meat products, at trace levels, based on reliable, accurate and sensitive methods.

So far, several techniques, mainly based on protein or DNA analysis, have been proposed for the identification of pork species in meat and/or processed meat products. Protein-based methods generally give satisfactory results for species identification in raw meats, but they present limitations when applied to thermally processed foods due to protein denaturation and alterations of specific epitopes (Rodríguez, García, González, Hernández, & Martín, 2005). Recently, the accurate analysis of biomarker peptides by liquid chromatography hyphenated with mass spectrometry detection has been suggested for animal species authentication in meat, including thermally processed products (Montowska, Alexander, Tucker, & Barrett, 2015; Sentandreu & Sentandreu, 2014; von Bargen, Dojahn, Waidelich, Humpf, & Brockmeyer, 2013). However, the high cost of such equipment and the need for specialised technicians are disadvantages to consider.

In recent works aiming at species identification in meat products, DNA molecules have been chosen as target compounds due to

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their higher stability when compared to proteins (Amaral, Meira, Oliveira, & Mafra, 2016; Fajardo et al., 2010). Moreover, DNA analysis based on polymerase chain reaction (PCR) technique presents a fast, sensitive, highly specific and less costly alternative, enabling the identification of species of origin even in complex processed foods (Amaral et al., 2015; Amaral, Santos, Melo, Oliveira, & Mafra, 2014; Bottero & Dalmasso, 2011; Soares, Amaral, Oliveira, & Mafra, 2013). In this regard, different approaches including PCR coupled to restriction fragment length polymorphism (PCR-RFLP) (Aida et al., 2005), multiplex PCR (Ali et al., 2015; Murugaiah et al., 2009), species-specific PCR (Che Man, Aida, Raha, & Son, 2007; Shabani et al., 2015), PCR-southern hybridization (Mutalib et al., 2015) and loop-mediated isothermal amplification (Ran et al., 2016) have been proposed for the specific identification of pork in products labelled as Halal. However, all these approaches are not able to provide quantitative information about the target DNA in foods. Quantification allows distinguishing between a deliberate adulteration for economic profit and a cross-contamination, which could be important both from a legal point of view and for quality control improvement in the meat industry. To date, few studies based on real-time PCR methods for pork species quantification in Halal products have been advanced, with some referring only to Halal gelatine or gelatine products (Cai, Gu, Scanlan, Ramatlapeng, & Lively, 2012; Demirhan, Ulca, & Senyuva, 2012) and not to Halal processed meat products. Farrokhi and Joozani (2011) used SYBR green I real-time PCR to detect pork DNA in commercially available meat extracts down to a level of 0.1 ng. However, the method was not normalised nor validated for the effective relative quantification of pork meat. Later, Ulca, Balta, Çagn, and Senyuva (2013) used a commercially available real-time PCR kit for pork identification in 42 Turkish processed meat products, which reached a level of 0.1% (w/w) of pork meat in meat mixtures. However, no quantitative approach was presented since results were only presented in terms of Cycle threshold (Ct) values.

As alternatives to SYBR green I, the third generation dyes, such as EvaGreen, offer enhanced fluorescence, allowing them to be used at higher concentrations, with greater signals, increased sensitivity and excellent stability without causing PCR inhibition (Wang, Chen, & Xu, 2006). The application of real-time PCR with EvaGreen dye has been reported in the identification of Cervidae species in feedstuff (Chen et al., 2009), beef and soybean in sausages (Safdar & Abasıyanık, 2013), beef and pork in sausages (Sakalar & Kaynak, 2016) and hare meat species (Santos et al., 2012).

The aim of this work was to develop and validate a novel specific and highly sensitive Evagreen real-time PCR system for the quantification of pork meat in processed meat products. For this purpose, model mixtures of beef meat spiked with known amounts of pork meat were used for in-house method development and validation. Since the analysed products were thermally processed foods, this study also aimed at evaluating the effect of thermal processing on the performance of the proposed real-time PCR method. Finally, to verify labelling compliance and evaluate the possible existence of fraudulent practices, the validated methodology was further applied to the analysis of several samples of commercial processed meat products, including Halal and regular products for which pork was not labelled as an ingredient.

2. Materials and methods

2.1. Binary model mixtures

Samples of pork (*Sus scrofa*) and cattle (*Bos taurus*) muscles were acquired from the retail market. Immediately after purchase, both meats were cut and the outside portions rejected. The samples were minced separately and reference binary mixtures containing 10.0%, 1.0%, 0.1%, 0.01%, 0.001%, 0.0005% and 0.0001% (w/w) of pork in beef meat were prepared to a final weight of 100 g by successive stepwise additions of minced beef meat. To evaluate the effect of thermal treatment, the mixtures of pork in beef meat were divided into two subsets, from which one was immediately stored at −20 °C, while the second was submitted to thermal processing by autoclaving during 15 min at 121 °C.

To avoid contaminations, all mixtures were homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and knives previously treated with DNA decontamination solution.

2.2. Validation mixtures and commercial samples

For method validation, blind mixtures were prepared in order to contain 6.0%, 4.0%, 2.5% and 0.25% (w/w) of pork in cattle meat. Similarly to the reference binary mixtures, validation mixtures were divided in two subsets, from which one was submitted to thermal processing by autoclaving during 15 min at 121 °C and the other was immediately stored at −20 °C.

A total of 15 Halal processed meat products, including canned sausages (n = 7), vacuum packed cocked sausages (n = 4) and canned mortadellas (n = 4), were acquired in a certified Halal market. Additionally, a total of 26 beef or poultry processed meat products, including hamburgers, meatballs, cannelloni, lasagnes and breaded meat, not labelled as Halal, but without declaring any pork derivative as ingredient, were acquired in local markets and used to assess the applicability of the method.

A wide range of non-target animal species and vegetable ingredients was also included in the study for specificity assays, namely chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), cattle (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), hare (*Lepus* spp.), partridge (*Alectoris rufa*), pheasant (*Phasianus colchinus*), duck (*Anas platyrhynchos*), quail (*Coturnix coturnix*), red deer (*Cervus elaphus*), rabbit (*Oryctolagus cuniculus*), ostrich (*Struthio camelus*), onion, garlic, parsley, white pepper, bay leaves, paprika, chilli, soybean, maize, rapeseed, sunflower, lupine, oat, barley, rye, almond, walnut, hazelnut, macadamia, pistachio, peanut, fava bean, pine nut, chestnut, wheat, rice, pumpkin, cashew, Brazil nut and pecan.

To avoid contaminations, all samples were homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and knives previously treated with DNA decontamination solution.

2.3. DNA extraction

DNA was extracted using the Wizard method with minor modifications as described by Soares et al. (2013). The extractions were performed in duplicate assays for each binary mixture and sample, being kept at −20 °C until further analysis.

2.4. DNA quantification and purity

The quality of extracted DNA was analysed by electrophoresis in a 1.0% agarose gel containing Gel Red 1x (Biotium, Hayward, CA, USA) for staining and carried out in STGB 1x (GRISP, Porto, Portugal) for 25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Yield and purity of extracts were assessed by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined