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SCIENCE DIRECT®
Food Control 16 (2005) 391–394
FOOD CONTROL
www.elsevier.com/locate/foodcont

# Duplex polymerase chain reaction for detection of pork meat in horse meat fresh sausages from Italian retail sources

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Received 16 February 2004; received in revised form 5 April 2004; accepted 6 April 2004

**Abstract**

Species identification in meat products represents an important subject in the field of modern food control according to the European Union, which has implemented a set of very strict procedures to label food. Thus, specific, sensitive and easy analytical methods for the species detection of food are necessary in order to verify the compliance with labelling requirements. A PCR-based assay for the detection of pork meat in horse fresh sausages was optimised and it was used to evaluate the presence of fraudulently added pork meat. The developed assay showed the presence of pork meat in 6/30 and the total absence of horse meat in 1/30 of the analyzed horse sausage samples.


*Keywords:* D-PCR; Species identification; Horse; Pork; Sausages

## 1. Introduction

The identification of meat products species is important to detect adulteration or fraudulent substitution and to preserve the consumers from the presence of unknown, less desiderable meat species for economic, religious and health reasons (Meyer, Höfelein, Lüthy, & Candrian, 1995). The European Union has implemented a set of very strict procedures for the labelling of food. Throughout the whole legislative procedure, the EU ensures the European consumers' right to be fully informed. Thus analytical methods for the species detection of food are necessary in order to verify compliance with labelling requirements. The control and detection of such foodstuffs may be based on protein detection tests using antibodies such as the enzyme-linked immunosorbent assay (ELISA)-test or DNA-based tests, such as polymerase chain reaction (PCR).

Methods based on protein detection such as electrophoresis, isoelectric focusing (IEF) are characterized by inaccuracy and may fail in species detection of processed meat products due to denaturation of soluble proteins during food processing. Moreover, the analysis by immunoassay, based on the use of antibodies raised against a specific protein, often presents cross-reaction with closely related species (Meyer, Candrian, & Lüthy, 1994).

The molecular methods based on PCR have been proposed as useful tools for the detection of several animal species (Colombo, Marchisio, Pizzini, & Cantoni, 2002; Guoli, Mingguang, Zhijiang, Hongsheng, & Qiang, 1998). They are highly specific, sensitive and characterized by a rapid processing time and low costs. However, the presence of inhibitors in foods, in particular in meat products, can prevent primer binding and diminish amplification efficiency, so that the extreme sensitivity achievable by PCR is often reduced when food is tested (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001).

In this paper a Duplex PCR (D-PCR) assay for the detection of pork meat in Italian horse fresh sausages, a meat product which requires cooking, was optimised to evaluate the presence of pork meat added fraudulently and consequently to verify the concordance with labels. The approach involves the extraction of DNA with a procedure in which total DNA is bound on a silica membrane followed by amplification of a fragment of the cytochrome *b* gene of mitochondrial DNA (mt DNA), a target used largely in species identification (Hsieh et al., 2001; Lau et al., 1998).

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doi:10.1016/j.foodcont.2004.04.004

## 2. Materials and methods

### 2.1. Controls

Horse (*Equus caballus*) and pig (*Sus scrofa domesticus*) DNA (BIOTOOLS B&M Labs, Madrid, Spain) were used as positive controls. Cow DNA (BIOTOOLS B&M Labs, Madrid, Spain) was used as negative control. A 100% pork fresh sausage, a 100% horse fresh sausage and sausages with different percentage of pork and horse meat, according to Matsunaga et al. (1999) were used as positive controls to optimise D-PCR.

### 2.2. DNA extraction

The sausage samples were subjected to DNA extraction using Tissue mini kit (QIAGEN, Hilden, Germany). 500 mg of fresh sausage were incubated with 10 ml of lysis buffer ATL (QIAGEN, Hilden, Germany) with 1 ml proteinase K (20 mg/ml) (QIAGEN, Hilden, Germany) at 56 °C overnight and then for 1 h at 70 °C with 10 ml Buffer AL (QIAGEN, Hilden, Germany). The mixture was centrifuged at 4000*g* for 2 min and ethanol was added to the transferred supernatant. The resulting mixture was applied to the QIAamp DNA spin column (QIAGEN, Hilden, Germany). The DNA bound to the column was washed in two centrifugation steps using two different wash buffers to improve the purity of the eluted DNA. The purified DNA was then eluted from the column in 80 μl of Elution Buffer (QIAGEN, Hilden, Germany). The DNA concentration and the purity of the eluate were measured by absorbance at 260 nm and by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm using a spectrophotometer DU-600 (Beckman, Fullerton, CA). The DNA eluted was used as template in the PCR assay.

### 2.3. Oligonucleotide primers

Primers **SIM** 5′-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3′, **PIG** 5′-GCT GAT AGT AGA TTT GTG ATG ACC GTA-3′ and **HOR** 5′-CTC AGA TTC ACT CGA CGA GGG TAG TA-3′, previously described and used in this study, were targeted a 398 bp fragment in pig and a 439 bp fragment in horse of mitochondrial cytochrome *b* genes (Matsunaga et al., 1999).

### 2.4. Duplex-PCR (D-PCR) assay

The reaction was performed in a final volume of 25 μl using 12.5 μl of HotStarTaq Master Mix (QIAGEN, Hilden, Germany and 0.5 μM of each primer and 20 ng of DNA. The D-PCR was processed in a Mastercycler 5332 (Eppendorf) with an initial denaturation step of 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension of 30 s at 72 °C and a final extension at 72 °C for 5 min.

### 2.5. PCR assay

The PCR assay was carried out to confirm the D-PCR results. The reaction was performed in a final volume of 25 μl using 12.5 μl of HotStarTaq Master Mix (QIAGEN, Hilden, Germany), 0.5 μM of each pig primers and 20 ng of DNA. The PCR was processed in a Mastercycler 5332 (Eppendorf) using D-PCR program. The same reaction was carried out using horse primers.

### 2.6. Amplified product detection

PCR amplified products were analyzed by electrophoresis on 2.5% (*w*/*v*) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X TBE buffer containing 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0 (USB, Cleveland, OH) and visualized by ethidium bromide staining and a UV transilluminator. A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) consisting of DNA fragments ranging in size from 3000 to 100 bp, was used as marker.

### 2.7. Testing samples

The procedures were used to test 30 fresh sausage declared made of pure horse meat collected from different markets in Apulia. The samples, stored at 4 °C, were transported to the laboratory and processed immediately. Samples found positive in PCR were subjected to sequence analysis to verify and confirm the specificity of the PCR products. Sequence analysis of PCR products was carried out by ABI PRISM 3100 (Applied Biosystem, Rome, Italy).

## 3. Results

### 3.1. DNA extraction

The approach followed for DNA extraction has allowed to extract DNA suitable for PCR amplification. The DNA extraction method, based on the binding of DNA to a silica matrix in presence of chaotropic agents, was considered effective and able to remove inhibitors, which could interfere with PCR reaction.

### 3.2. D-PCR

The study confirmed the primer specificity and detection limit according to Matsunaga et al. (1999). None of the bovine DNA samples gave positive results. D-PCR procedure to amplify mitochondrial cytochrome *b* gene from pig and horse DNA indicates the presence