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IDENTIFICATION OF ANIMAL SPECIES IN HEAT TREATED MUSCLE HOMOGENATES USING THE POLYMERASE CHAIN REACTION

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The possibility of identification of animal species in pasteurized and sterilized muscle homogenates was studied. Homogenates consisted of beef, pork and chicken meat, mixed in equal amounts. Nitrite salt and polyphosphates were also added. Heat treatment included pasteurization at temperatures of 80°C, 90°C and 100°C, while sterilization was performed at temperatures of 110°C and 121°C, respectively. Animal mitochondrial DNA was isolated from the samples and the target gene was subsequently amplified by PCR. The presence of species-specific bands indicated respective animal species in homogenates. The results showed that it was possible to identify the most common domestic animal species in heat treated muscle homogenates.

Key words: meat, animal species, identification, PCR

INTRODUCTION

During the last several years a number of analytical methods such as ELISA, capillary electrophoresis and polymerase chain reaction (PCR) have been developed in order to enable an accurate identification of species in meat products. Methods based on identification of species-specific proteins proved not to be reliable, since a number of false-positive results was observed. They were also time-consuming since multiple protein bands extended beyond each other making identification quite ambiguous. This was particularly the case with heat treated products. False-positive results occur due to instability of proteins at temperatures above 56°C since above this temperature they undergo denaturation (Kang'ethe *et al*., 1986). DNA is a much better substrate to use in species identification. It is a heat stable molecule which can withstand temperatures of pasteurization and sterilization of meat products. In the beginning of 1990's DNA-based analytics used oligonucleotide probe hybridization assays or restriction fragment length polymorphism as tools for species identification. However, since 2001 most researchers have used species-specific primers; short oligonucleotide fragments flanking genes in the DNA molecule, in order to improve speed and accuracy of identification (Calvo *et al*., 2001). In a comparative

study, Meyer, Candrian and Luthy (1994) reported that pork at a 2% level was identified by PCR in meat products after cooking at 121°C for 10 min while only 50% levels were identified by ELISA in the same product. Hopwood *et al*. (1999) detected chicken meat after cooking at 80°C, 100°C, or 120°C for 30 min by PCR. Matsunaga *et al*. (1999) studied the identification of pork, chicken, lamb, goat, and horse meat using PCR after cooking at 100°C and 120°C for 30 min. With the exception of horse meat cooked at 120°C, all other samples were identified successfully. Arslan, Ilhak and Calicioglu (2006) exposed beef meat to the temperatures ranging from 90°C-200°C (autoclaving, frying and roasting) for a period of up to 150 minutes and reported that they succeeded in DNA identification, although meat had lost its sensory attributes and in cases of frying even became charred.

Due to recently adopted food safety and food labelling regulations in Serbia lying down that all food business operators should declare meat species in meat products to consumers we have investigated the possibility of species identification in heat treated muscle homogenates using PCR. The content of muscle homogenates actually represented stuffing of boiled sausages. Muscle homogenates were prepared in a local mid-size meat plant and were exposed to temperatures of pasteurization and sterilization.

MATERIAL AND METHODS

Preparation of muscle homogenates

Muscle homogenates were prepared in a local mid-sized plant. Overall weight of homogenates yielded 100 kg. The volume consisted of equal amounts of beef, pork and chicken meat (25 kg each), 25 kg of ice, 2 kg of nitrite salt (99.6% NaCl + 0.4% $NaNO_2$) and 0.5 kg of polyphosphate. After preparation, homogenates were stuffed into polyamide casings, as well as in cans and designated by numbers from 1 to 15.

Heat treatment

Muscle homogenates which were stuffed into polyamide casings were thermally treated at temperatures of 80°C, 90°C and 100°C, respectively. Internal temperatures of homogenates during pasteurization were measured by K type thermocouple (HI 9057 KJT thermocouple, Hanna Instruments, Portugal). Pasteurization lasted until the desired temperature was attained in the "coldest spot" of the homogenate. Canned muscle homogenates were subjected to temperatures of 110°C and 121°C, respectively at a pressure of 1.3 bars, during 45 minutes.

Three samples were subjected to each temperature and the entire experiment was tripled making a total of 45 (3×5×3) samples investigated.

DNA isolation

DNA was isolated from muscle homogenates using commercial DNeasy Tissue Kit (Quiagen GmbH, Germany). After heat treatment, muscle homogenates were homogenized once more at 25000 rpm using a high speed

homogenizer (DI25, Ika Werke GmbH, Germany). Afterwards 25 mg of each homogenate was placed in a 2 mL microcentrifuge tube and 180 μL of Buffer ATL was added. After vortexing 20 μL of Proteinase K was added to the solution. Suspensions were incubated at 55^{o}C for 3 hours in order to enable complete lysis of myocytes. After incubation 200 μL of Buffer AL was added and the mixture was subjected to 70^{o}C for 10 minutes. The suspension was vortexed and 200 μL of ethanol (96%) was added and the entire volume (approx. 500 μL) was pipetted into special DNeasy Mini Spin columns. The tubes were centrifuged at 8000 rpm and flow-through was discarded. The same procedure was repeated using 500 μL of buffers AW1, AW2 and 200 μL buffer AE. The last eluate, originating from buffer AE, was collected in a special tube since it contained DNA. Isolation lasted for 4 hours. Final DNA concentration and purity were measured using spectrophotometer (Jenway 6405, UK) by calculating A_{260} value and A_{260}/A_{280} ratio. DNA samples displaying A_{260}/A_{280} ratio less than 1.7 were subjected to repeated elution. DNA was kept at 4^{o}C until amplification of the target gene occured.

PCR

A total volume of 50 μL of the reaction mixture was prepared in an eppendorf tube. Commercial Biofood Mixed Kit (Biotools, S.A. Spain) was used. Reaction mixture consisted of 25 μL Master Mix (10 mM Tris-HCl pH 9.0, 50 mM KCl, <10% glycerol, <0.001% dNTP's and primers flanking species-specific regions of a cytochrome b mtDNA), 2 μL of 50 mM $MgCl_2$, 5U of Taq DNA-polymerase and 10 μL of target DNA. Besides target DNA, three controls DNA (chicken, beef, pork) were also put into reaction mixtures as a positive control. Thermocycler (Flexigene 412, Techne, GmbH, Germany) was programmed for 35 cycles. After initial denaturation at 94^{o}C for 1 minute and 30 seconds, each cycle consisted of denaturation at 94^{o}C for 10 seconds followed by annealing at 60^{o}C for 30 seconds and elongation hold at 72^{o}C for 40 seconds. At the end of the entire process the mixture was subjected to 72^{o}C for 3 minutes in order to finalize the elongation of remaining DNA molecules. Prior to gel electrophoresis the mixture was stored at -20^{o}C.

Sensitivity test

In order to establish the sensitivity of assay, three mixtures of DNA were prepared. Each mixture consisted of equal amounts of cow, pig and chicken DNA diluted to final concentrations of 5%, 1% and 0,5%, respectively. The DNA dilutions were subjected to PCR amplification conditions as for regular samples.

Gel electrophoresis

PCR products (15 μL) mixed with 5 μL loading buffer (6×Orange Dye, Fermentas, Lithuania) were electrophoresed at 120V for 30 minutes through 2% (w/v) agarose (MB Agarose, Biotools, S.A. Spain) gel in 1×TBE, containing 0.5 μg/mL ethidium bromide (Sigma, Germany). Ladder (O'Range 50 bp) was placed (10 μL) to run alongside the samples. Bands were visualized on a UVP transluminator and photographed using a digital imaging system (Olympus, Japan).

RESULTS AND DISCUSSION

Average temperatures attained in the "coldest spot" of muscle homogenates during pasteurization were 75°C, 87.5°C and 98.4°C, respectively.

Results of sensitivity testing are shown on Figure 1. Bands of all three species are clearly visible at the concentration of 5% and 1%, however considerable fainting occurred at DNA concentration of 0.5%. PCR amplicons of heat treated muscle homogenates are exhibited on Figures 2 and 3. The chicken DNA positive control generated one product sized 227 bp, beef DNA positive control generated one product sized 278 bp and pork DNA positive control generated one product sized 398 bp. At 80°C, all three species were successfully identified; displaying distinct bands for all three species DNA products. Equal results occurred with homogenates processed at 90°C and 100°C, respectively. Canned muscle homogenates exhibited clear bands for chicken and pork DNA product while the beef DNA products occurred as a considerably faint band.

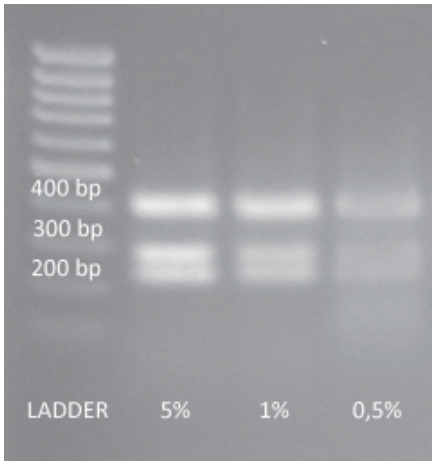

Figure 1. Results of the sensitivity test

Our results are in agreement with those obtained by Arslan, Ilhak and Calicioglu (2006). It is possible accurately to identify species in pasteurized and sterilized meat products, however faint beef DNA bands obtained from sterilized products indicate that primers used for amplification of beef mtDNA flank smaller DNA fragment, so that high temperatures and pressure brake down already damaged beef DNA. Established sensitivity of 0.5% of target species DNA was lower compared to findings of Kesmen *et al*. (2007) which established the sensitivity to be 0.1% for samples treated with temperatures up to 100°C up to 30 minutes. However, it should be taken into account that Kesmen *et al*. (2007) established sensitivity on the basis of DNA dilution, while we established sensitivity on the basis of meat fractions within the homogenates.

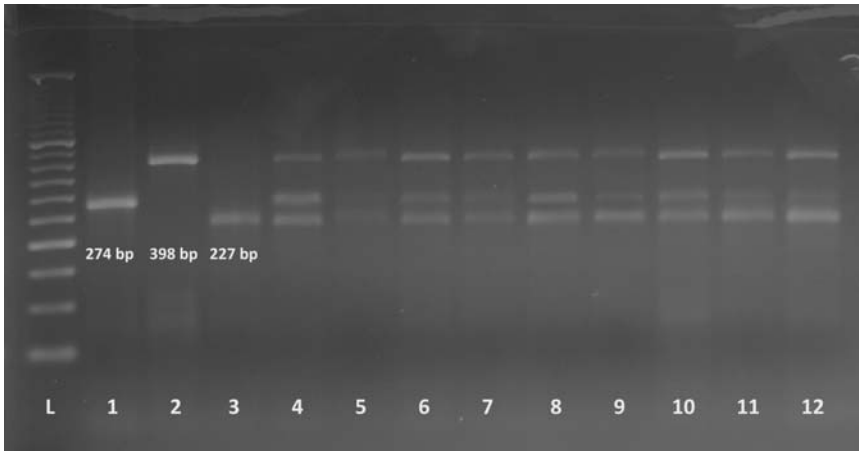

Figure 2. PCR products of pasteurized muscle homogenates. L-Ladder, 1-Beef positive control, 2-Pork positive control, 3-Chicken positive control, 4-6-Muscle homogenates pasteurized at 80°C, 7-9-Muscle homogenates pasteurized at 90°C, 10-12-Muscle homogenates pasteurized at 100°C

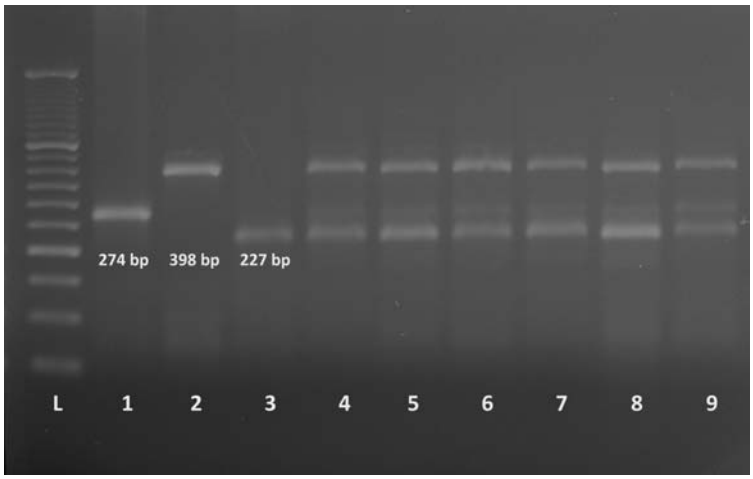

Figure 3. PCR products of sterilized muscle homogenates. L-Ladder, 1-Beef positive control, 2-Pork positive control, 3-Chicken positive control, 4-6-Muscle homogenates sterilized at 110°C, 7-9-Muscle homogenates sterilized at 121°C

In conclusion can be elicited that PCR can efficiently replace protein-based analytical methods due to low expenses and time savings. Due to high processing temperatures and subsequent excessive protein denaturation further investigations should be performed in order to comparatively establish efficiency of ELISA-based methods.

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IDENTIFIKACIJA VRSTA ŽIVOTINJA U TERMIČKI TRETIRANIM MIŠIĆNIM HOMOGENATIMA KORIŠĆENJEM PCR METODE

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SADRŽAJ

U radu je ispitivana mogućnost identifikacije vrsta životinja u pasterizovanim i sterilizovanim mišićnim homogenatima. Homogenati su se sastojali od govođeg, svinjskog i pilećeg mesa pomešanog u jednakim odnosima. Pored mesa, u homogenate su dodati nitritna so i polifosfati kako bi se što vernije simulirali uobičajeni sastojci nadeva. Termički tretman podrazumevao je pasterizaciju pri temperaturama od 80°C, 90°C i 100°C i sterilizaciju pri temperaturama od 110°C i 121°C. Iz pripremljenih uzoraka izolovana je mitohondrijalna DNK i amplifikovan je mitohondrijalni citohrom B gen. Prisustvo specis-specifičnih traka indikovalo je i prisustvo mesa specifične vrste životinja u uzorcima. Rezultati ispitivanja ukazuju da je PCR metodom moguće identifikovati najčešće vrste životinja u termički tretiranim mišićnim homogenatima.