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Determination of pig sex in meat and meat products using multiplex real time-PCR

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ABSTRACT

For specific production lines, European retail companies demand exclusively female pork meat. To control the quality of their suppliers the identification and a quantitative detection of the animal sex origin of the meat is therefore of importance for meat processors. To enable a fast and reliable detection of male pig meat, a real time-PCR-system was designed in the present study. This was based on the genes AMEL-X and AMEL-Y. The real time-PCR assay allowed the detection of male pig meat at a concentration of 1% yielding a detection probability of 100% while the detection probability investigating meat samples containing 0.1% male pig meat was 44.4%. The analytic sensitivity of this system was assessed to be <5 pg DNA per PCR reaction. The assessment of the accuracy of the real time-PCR assay to correctly identify sex individuals was investigated with 62 pigs including males (n = 29) and females (n = 33) belonging to different breeds/lines. With the newly designed test all analysed animals were correctly sexed. No amplification was obtained with cow, goat, sheep, turkey and chicken genomic DNA. The presented assay can be used for sex diagnosis, for the detection of male pig meat and for meat quality control.

1. Introduction

Boar meat can contain skatole and androstenone that might result in an unpleasant odour (sexual odour) and taste of meat (Claus, Weiler, & Herzog, 1994). Considering the regulation (EC) 854/04 meat with pronounced sexual odour has to be declared unfit for human consumption. Pork producers obviate these problems during meat inspection at the slaughterhouse by castrating male piglets (castrates) (Anonymous, 2004). However, as castration using anaesthesia and/or analgesia may only be done by veterinarians these surgeries are expensive and ineffective. Therefore the directive 2008/120/EC (formerly 1991/630/EC) includes an exception giving the permission to specifically trained persons to castrate piglets not older than seven days without anaesthesia (Anonymous, 2008). This regulation is criticised by animal welfare organisations and some retail companies. These companies reacted by excluding meat from pigs castrated without anaesthesia and/or selling only pork from sows. However, immunocastration as alternative to castration without anaesthesia is less accepted by consumers (Fuchs et al., 2009). Slaughter of boars might be done, if food processor and competent authorities verify that no meat with unacceptable odour and taste is sold. However, some retail companies favour sow meat to fully exclude sexual odour for specific production lines. Therefore, to control suppliers a fast and precise method for sex determination of meat and meat products is necessary. Sex determination in itself does not solve the boar taint problem and exclusion of male pig meat from specific production lines includes also meat which is not prohibited by EC-regulation. However, for specific products retail companies can choose to avoid ethical conflicts with consumers. The aim of the present investigation was to develop and evaluate a quantitative real time PCR for gender differentiation in meat and meat products which can be used in food quality analysis.

2. Material and methods

2.1. Investigated samples and DNA extraction

The longissimus muscle samples used in the present study were obtained from 62 domestic pigs (Sus scrofa domestica). These frozen samples included males (n = 29) and females (n = 33) belonging to five different breeds/lines: Pietrain MHS homozygous negative (PiNN, n = 18); Pietrain MHS homozygous positive (PiPP, n = 12); Duroc (Du, n = 16) as well as the crossbreeds/ lines of Du and PiNN (DuPi, n = 14) and Pi x F1 (n = 2). The samples were coded and tested by the laboratory persons without origin information. For the exclusivity test cow, sheep, goat, turkey and chicken DNA were tested in parallel. Genomic DNA was extracted from the tissues samples using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 25 mg of the tissue sample was lysed, followed by binding of the DNA to the spin-column (Qiagen). After washing steps the DNA was eluted with 100 µl elution buffer, the quantity of the DNA was measured using Qubi™ Flourometer

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Table 1
Oligonucleotide primers and fluorogenic probe sequences used in this study.

Designation	Sequence	Positions AB091791, 2	Melting temp. (°C)
AMEL-X-S (forward)	5′-gATTAACTgAgCACAggggTgg-3′	4964–4985	59.4 °C
AMEL-X-A (reverse)	5′-ggAACATgTATgCTgggTAggAgA-3′	5129–5106	59.1 °C
AMEL-Y-F (forward)	5′-TgTATTTCATgTTATCAgATgCAgAgAT-3′	753–780	58 °C
AMEL-Y-R (reverse)	5′-CCAAAgAATgTTATTgTggAAgCA-3′	802–826	59 °C
AMEL-X-TaqMan® probe	YAK-AgAgCAACgAAATggTTTTCC-BBQ	5079–5059	55.6 °C
AMEL-Y-TaqMan®mgb probe	FAM-CCACATTTCTCATACTTT -MGB-NFQ	783–799	68 °C

(Invitrogen, Darmstadt, Germany). The DNA were stored at 4 °C or used as a DNA template (2.5 µl) for the real time PCR.

2.2. Oligonucleotide primers and probes

The developed real time-PCR was based on the reference sequence of AMELX and AMELY gene published in the National Centre for Biotechnology (NCBI) GenBank under accession no. AB091791 and AB091792. In addition alignments were performed containing the sequence entries of similar sequences by comparative search using NCBI Genbank. The sequence data were further studied with the computer program MegAlign Lasergene, (DNASTAR Inc. Wisconsin, USA).

The primers were designed using the computer program Primer Express® Version 0.2 (Applied Biosystems, Darmstadt, Germany). Two primer pairs were selected to be specific for AMEL-X and AMEL-Y. A TaqMan® probe labelled at the 5′-end with 5′ Yakima Yellow® (YAK) was designed for AMEL-X, the 3′-end contained non-fluorescent Quencher BlackBerry® Quencher (BBQ). For AMEL-Y target gene a TaqMan®-Minor Groove Binder (mgb) probe labelled at the 5′-end with 6-carboxyfluorescein (FAM) was selected, the 3′-end contained Non-Fluorescent Quencher (NFQ). All oligonucleotide primers and the AMEL-X -TaqMan® probe were synthesised by TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany, while the AMEL-Y-TaqMan®mgb probe was synthesised by Applied Biosystems. The oligonucleotide sequences were submitted to the NCBI Genbank for specificity testing including broad range and comparative genome Basic Local Alignment Search Tool (BLAST) analysis. The sequences of the oligonucleotide primer and fluorogenic probes, their positions and the melting temperatures are summarised in Table 1.

2.3. Real time-PCR assay

For the real time-PCR assay using TaqMan® probes the optimised 25 µl multiplex-PCR mixture contained end concentration of 0.4 µM of the primers AMEL-X-S and AMEL-X-A, 0.2 µM of the primers AMEL-Y-F and AMEL-Y-R, 0.2 µM of the target gene probes AMEL-X and AMEL-Y and 12.5 µl of the 2× Fast qPCR™ MasterMix (Eurogentec Deutschland GmBH, Köln, Germany) and a 2.5 µl aliquot of the DNA sample. The PCRs were performed in a 96 well plate format with the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). Thermal cycling conditions comprised a Uracil-N-glycosylase (UNG) step at 50 °C for 2 min, a Hot Start DNA Polymerase activation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 3 s, an annealing at 63 °C for 30 s, and an extension at 72 °C for 10 s. Each quantitative measurement was performed in triplicate and the threshold cycle /crossing point (C_p) (the fractional cycle number at which the amount of amplified target reached a fixed threshold) was determined automatically by using Abs. Quant/2nd Derivative max analysis setup.

2.4. Melting curve analysis

To verify the specificity of the amplified products and homology of the sequences contained among the breeds/lines investigated the pig meat samples were also tested with a Sybr Green based detection system. For this LightCycler® 480 DNA SYBR Green I Master (Roche) was used with the standard LightCycler® 480 thermo cycler program followed by melting curve analysis.

2.5. Construction of standard curve and assessment of amplification efficiency

Standard curves were generated from pig DNA isolated from male and female. The amount of the DNA was estimated by using Qubi™ Flourometer. Serial dilutions (10^0–10^{-4}) were prepared using TE buffer (pH 8.0). The real-time PCR amplification of the serially diluted pig DNA was performed using the same PCR conditions mentioned above. The threshold cycle (C_p) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). The C_p values were subsequently used to calculate and plot a linear regression line by plotting the logarithm of template concentration (X-axis) against the corresponding threshold cycle (Y-axis). All the reactions were run in triplicate and the C_p were averaged from the values obtained in each reaction. A standard curve was then constructed by plotting the C_p of known concentration of each standard sample. The quality of the standard curve can be judged from the slope value. The slope of the line can be used to determine the efficiency of the target amplification (E) using the equation $E = (10^{-1/\text{slope}})$-1.

2.6. Internal reference material (IRM) and limit of detection (LOD) of male pig meat

To determine the detection limit, different internal reference materials (IRM) were prepared. Meat mixtures of 100 g of female meat were prepared with 10%, 5%, 2%, 1%, 0.1% and 0.01% of male pig meat. The mixtures were homogenised by using Grindomix (Retsch, Haan, Germany). The DNA was isolated in triplicate from each concentration and investigated in three runs with the real time PCR conditions mentioned above.

3. Results

3.1. Specificity and the analytic sensitivity of the real time-PCR

Using the primer and oligonucleotide probe set designed for AMEL-X and AMEL-Y all 62 pig meat samples were identified correctly. No amplification of the 5 non-pig meat samples was observed. Treshold cycle values (C_p) for various DNA extractions from different pig breeds/line ranged between 28.97 and 34.74. No significant sequence differences for the generated AMEL-X and AMEL-Y PCR products of different breed /lines were obtained by melting curve analysis using Sybr Green analysis. The melting temperature was 82 °C for the AMEL-X and 73 °C for the AMEL-Y amplicon (Fig. 1).

The amplification rate was calculated on the basis of a linear regression slope of a dilution row. The amount of the DNA ranging from 138 ng/µl (10^0) to 13.8 pg/µl (10^{-4}) for male pig meat DNA while the female DNA quantity were from 20 ng/µl (10^0) to 2 pg/µl. (10^{-4}) It was possible to obtain a series of amplification plots and to determine their absolute C_p values. The analytic sensitivity of this