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A novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of meat and commercial meat products from game birds.

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Abstract

Species-specific real-time polymerase chain reaction (PCR) assays using TaqMan probes have been developed for verifying the labeling of meat and commercial meat products from game birds including quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock and song thrush species. The method combines the use of species-specific primers and TaqMan probes that amplify small fragments (amplicons <150 base pairs) of the mitochondrial 12S rRNA gene, and an endogenous control primer pair that amplifies a 141 bp fragment of the nuclear 18S rRNA gene from eukaryotic DNA. Analysis of experimental raw and heat-treated binary mixtures as well as of commercial meat products from the target species, demonstrated the suitability of the assay for the detection of the target DNAs.

Keywords: species identification; 12S rRNA; 18S rRNA; game birds; real-time PCR; TaqMan

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47 Introduction

48 Traceability of food products has become a priority for governments of the
49 developed countries, due to consumer demand for comprehensive and integrated
50 food quality policies. In this context, food labeling legislation has become an
51 important issue because of the potential direct impact of labels on consumer
52 decision-making (Caporale et al. 2001; Verbeke and Ward, 2006). Meat products
53 sold for public consumption must be accurately labeled regarding species content
54 and thus, any ambiguity in the labeling practices of commercial suppliers is
55 unacceptable. The fraudulent mislabeling of meat is a widespread problem,
56 particularly with high added-value meat species commanding a premium price
57 (Woolfe and Primrose, 2004). This has resulted in a need for tests which will reliably
58 identify the species of meat present in a food sample and which also must be
59 sensitive and robust enough to be applied to complex food matrices (Chisholm et al.
60 2005).

61 A particular type of product in which adulteration may take place is game bird
62 meat. This type of meat can be found in gourmet foods that tend to be eaten on
63 special occasions and because of their specialized nature, command high prices. As
64 consumer's tastes and choices broaden, the demand for less common game type
65 meats is likely to grow, together with the need for reliable and rapid methods to
66 ensure the accurate labeling of these products (Chisholm et al. 2008). Such methods
67 must be developed to protect both consumers and producers from frauds, but also to
68 prevent over-exploitation and illegal trafficking of game meat products through the
69 food market (Fajardo et al. 2007).

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To accomplish meat authentication, a range of analytical approaches have been tackled, broadly based on detecting either protein or DNA molecules (Teletchea et al. 2005). In comparison with proteins, DNA-based methods have proved to be more reliable because of the stability of DNA under the conditions associated with the high temperature, pressures and chemical treatments used in the preparation of some meat products (Rodríguez et al. 2004). DNA-based methods have commonly been based around the use of conventional PCR with species-specific primers (Di Pinto et al. 2005; Che Man et al. 2007; Kesmen et al. 2007; Martín et al. 2007a; Rojas et al. 2009). However, this technique presents limitations such as the requirement of post-amplification handling of PCR products, thereby extending the risk of laboratory contamination and false-positive results, and the dependence on agarose gel electrophoresis for analysis of the products. More recently published reports have focused on the use of specific primers in real-time PCR (Dooley et al. 2004; Tanabe et al. 2007; Fajardo et al. 2008a). This technique has provided sensitive and safe solutions by monitoring PCR products continuously using fluorescent markers that eliminate the need for post-PCR processing steps, easing automation of the technique and large scale sample processing (Zhang et al. 2007).

In this paper, we describe the development of a real-time PCR approach for the detection of quail (*Coturnix coturnix*), pheasant (*Phasianus colchicus*), partridge (*Alectoris* spp), guinea fowl (*Numida meleagris*), pigeon (*Columba* spp), Eurasian woodcock (*Scolopax rusticola*), and song thrush (*Turdus philomelos*) DNAs in raw and heat-treated meat mixtures, and commercial meat products using TaqMan fluorogenic probes as detection platform. The assay targets species-specific sequences on the 12S rRNA gene and makes use of a eukaryotic system based on an 18S rRNA fragment as endogenous control.

95 **Material and methods**

96 ***Selection and preparation of meat samples***

97 Authentic muscle samples of quail (*Coturnix coturnix*), pheasant (*Phasianus*
98 *colchicus*), red-legged partridge (*Alectoris rufa*), guinea fowl (*Numida meleagris*),
99 Eurasian woodcock (*Scolopax rusticola*), woodpigeon (*Columba palumbus*), and
100 song thrush (*Turdus philomelos*), were provided by Antonio de Miguel (Madrid,
101 Spain). Red-legged partridge samples were also obtained from the Estación
102 Biológica de Doñana (Sevilla, Spain) and from Hermanos Sainz (Madrid, Spain).
103 Chukar partridge (*Alectoris chukar*) meat samples were provided by Hermanos Sainz
104 (Madrid, Spain). Barbary partridge (*Alectoris barbara*) meat samples were obtained
105 from the Department of Animal Production (Facultad de Veterinaria, Universidad
106 Complutense de Madrid). Common pigeon (*Columba livia*) meat samples were
107 provided by Industria del Pichón Bravío (Valladolid, Spain). Capercaillie (*Tetrao*
108 *urogallus*) meat samples were obtained from the Department of Animal Pathology
109 (Facultad de Veterinaria, Universidad Autónoma de Barcelona, Spain). Chicken
110 (*Gallus gallus*), turkey (*Meleagris gallopavo*), Muscovy duck (*Cairina moschata*), and
111 goose (*Anser anser*) meat samples were obtained from several local abattoirs and
112 retail markers (Madrid, Spain). Muscle samples from cattle (*Bos taurus*), sheep (*Ovis*
113 *aries*), goat (*Capra hircus*), swine (*Sus scrofa domestica*), red-deer (*Cervus elaphus*),
114 fallow-deer (*Dama dama*), roe-deer (*Capreolus capreolus*), chamois (*Rupicapra*
115 *rupicapra*), mouflon (*Ovis ammon*), and pyrenean ibex (*Capra pyrenaica*) were also
116 obtained from several Spanish abattoirs and meat-cutting installations. All specimens
117 were morphologically identified by professional veterinaries before obtaining the
118 samples. Fresh muscle portions from the selected specimens were processed
119 immediately or stored frozen at -20 °C until use.

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Binary mixtures of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, or song thrush, in a chicken meat matrix were prepared using raw muscles from the selected species. For each meat mixture, six different percentages containing 50%, 25%, 10%, 5%, 1%, and 0.1% (wt/wt) of the target species were prepared to a final weight of 100 g. Forty ml of sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH₂PO₄, 8.09 mM Na₂HPO₄ · 12H₂O, and 2.6 mM KCl, pH 7.2) were added to the binary mixtures and they were homogenized with a blender (Sunbeam Oster, Florida, USA). Fifty grams of each mixture were sterilized at 121 °C for 20 min to check the effect of thermal treatments on the technique's ability to identify the target species. Raw and sterilized binary meat mixtures were processed directly or stored at -20 °C until use.

Several commercial meat products from quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock and song thrush species purchased from different retail markets (Spain) were also included in the analysis.

DNA extraction

Total DNA was extracted from meat using a Wizard® DNA Clean-up System kit (Promega Corp., Madison, WI) as described in a previous work (Rojas et al. 2008). DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, DE).

Primers and oligonucleotide probes design

Based upon detailed analysis and comparison of the alignment of 12S rRNA sequences from quail, pheasant, red-legged partridge, chukar partridge, barbary partridge, guinea fowl, woodpigeon, Eurasian woodcock, song thrush, capercaillie, chicken, turkey, Muscovy duck and goose (Rojas et al. 2009), seven species-specific

144 primer pairs were designed: 12SCOT-FW/12SCOT-REV, 12SPHA-FW/12SPHA-
145 REV, 12SALEC-FW/12SALEC-REV, 12SNUM-FW/12SNUM-REV, 12SCOLU-
146 FW/12SCOLU-REV, 12SSCO-FW/12SSCO-REV, and 12STUR-FW/12STUR-REV.
147 They were expected to yield specific fragments of 129, 113, 141, 130, 133, 138, and
148 110 bp in the 12S rRNA gene of quail, pheasant, partridge, guinea fowl, pigeon,
149 Eurasian woodcock and song thrush, respectively. Quail, pheasant, partridge, and
150 guinea fowl primers were designed in a previous work (Rojas et al. 2009) whereas
151 pigeon, Eurasian woodcock, and song thrush primers were designed in this work.
152 Besides, a conserved 141 bp fragment of the 18S rRNA gene flanked by 18SpEUDIR
153 and 18SpEUINV primers was used as endogenous control of the assay.

154 The EMMA program included in the EMBOSS software package version 2.0
155 and Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster
156 City, CA) were used for sequence alignment and primer design.

157 Due to the high degree of sequence similarity between the species selected,
158 only limited regions could be used to design species-specific primers. The different
159 position of the species-specific primers designed made not possible the use of a
160 common probe for the seven species selected. Thus, taking into account the position
161 of the primer pairs designed, species-specific TaqMan probes were designed for the
162 detection of quail (QUAIL-TM), pheasant (PHEASANT-TM), Eurasian woodcock
163 (WOODCOCK-TM), and song thrush (THRUSH-TM) respectively, whereas a
164 common probe (BIRDS-TM) was designed for the detection of partridge, guinea fowl,
165 and pigeon species. Besides, another TaqMan probe (18SPROBE) based on
166 conserved 18S rRNA gene sequences available in the Genbank/EMBL database for
167 various avian and mammal species was used together with 18SpEUDIR and
168 18SpEUINV primers as endogenous control PCR system.

TaqMan probes were designed and synthesized by TibMolBiol (Berlin, Germany). The TaqMan probes were aimed to anneal within the gene fragment generated by amplification of the corresponding target and were labeled on the 5' end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and on the 3' end with the blackberry (BBQ) fluorescent quencher.

In addition, species-specific primers developed in previous works (Martín et al. 2007b, 2007c, 2008) for chicken, turkey, duck, cattle, and swine were used in conventional PCR assays for the analysis of complex meat products from the target species in order to verify the composition stated in the labels.

The sequences and description of the primers and probes used in this work are summarized in Table 1.

PCR conditions

Real-time PCR was run under generic cycling conditions. The optimum PCR concentrations of primers yielding highest endpoint fluorescence and the lowest C_p were experimentally determined for each set of primers. Optimum concentrations per reaction for each primer are shown in Table 1. The PCR reactions were carried out using the LightCycler® TaqMan® Master (Roche Diagnostics GmbH, Mannheim, Germany), 2 pmol of each TaqMan probe (TibMolBiol), and 5 ng of DNA. Amplification reactions were performed in a total reaction volume of 10 µL in a glass capillary tube and were run on the LightCycler® 2.0 Instrument (Roche Applied Science, Pensberg, Germany) with the following program: 10 min at 95 °C (denaturation and Taq polymerase activation), an amplification program of 35 cycles at 95 °C for 10 s, 65 °C (for quail, partridge, guinea fowl, and Eurasian woodcock), 63 °C (for pigeon, and song thrush), or 60 °C (for pheasant) for 30 s,

and 72°C for 1 s. Samples were then cooled to 40 °C for 30 s. The same program was used to amplify each of the species-specific systems along with the endogenous control PCR fragment. The crossing point value (C_p), which refers to the cycle number where the sample's fluorescence significantly increases above the background level of fluorescence, was calculated automatically by the LightCycler® software as the first maximum of the second derivative of the curve. Unless otherwise indicated, all real-time PCR reactions were carried out in duplicate.

Construction of standard curves and data analysis

In order to check the ability of the real-time PCR method for quantitative detection, standard curves were constructed using different percentages (50, 25, 10, 5, 1, and 0.1 %) of raw and sterilized binary meat mixtures from quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush.

C_p values obtained from each sample with the corresponding species-specific system (C_{pSP}) were normalized according to the following equation:

$$C_p \text{ SPS} = C_{pEU} * C_{pSP} / C_{pEUS}$$

where C_{pSPS} is the normalized C_p value of the sample analyzed with the corresponding specific PCR system, C_{pEU} is the C_p average value of the standard samples (binary mixtures) in the endogenous PCR system, and C_{pEUS} is the C_p value of the sample analyzed with the endogenous PCR system.

In quantification analysis, a standard curve of standard samples plotted against their crossing point is used to determine the concentration of unknown samples. The amount of target DNA in an unknown sample can be measured by interpolation of the normalized C_p value obtained in the unknown sample in the

corresponding standard curve of C_p values generated from known DNA percentages of the target species.

Besides, the correlation between the variables, crossing point (C_p) and concentration ($[]$) is semilogarithmic:

$$C_p = b \log [] + a$$

Where b is the slope and a is the intercept.

Linearity test, sensitivity, accuracy and precision parameters of the species-specific real-time PCR systems were evaluated according to previously described methods (Camacho et al. 1993; ICH Harmonized Tripartite Guideline. 2005). To carry out the validation of the real-time PCR technique developed in this work, three separate DNA extractions of each mixture percentage were assayed in different days, using two replicates of each sample. Precision of the assay was evaluated by comparing the coefficient of variation (CV) values obtained in three different days versus those obtained in the same day, to asses the influence of random events (day of analysis, analyst, equipment, etc.) in the data obtained from the binary mixtures.

Results and discussion

Real-time PCR system set-up

The primary objective of the PCR system setup was to establish an adequate marker for game bird species identification. As target for real-time PCR, a fragment of the mitochondrial 12S rRNA gene was selected. This mitochondrial gene was chosen because it shows an appropriate degree of intra- and inter-species variability, and because of the high number of copies found per cell. The presence of a high copy number of mitochondrial DNA molecules in a cell significantly increases the

241 sensitivity of the PCR assay when the tissue is subjected to extreme processing
242 conditions, such as sterilization (Girish et al. 2004).

243 To develop a robust method for detection of quail, pheasant, partridge, guinea
244 fowl, pigeon, Eurasian woodcock, and song thrush species in meat mixtures and
245 commercial meat products, seven species-specific primers and an eukaryotic primer
246 set (endogenous control) were combined in a real-time PCR method. The use of an
247 18S rRNA conserved region as an endogenous control has several purposes: a) it
248 provides a positive control on the total PCR-amplifiable eukaryotic DNA present in
249 the sample because factors affecting variation of target DNA amplification such as
250 PCR inhibition, degradation and quality of the DNA recovered from a sample can be
251 taken into account; b) it traces the presence of DNA that has not been amplified by
252 the species-specific detectors; c) by comparison of species-specific versus
253 endogenous control signal obtained from the samples, the inaccuracies caused by
254 the use of standards and unknowns can be reduced.

255 With respect to the species-specific systems, quail, pheasant, partridge,
256 guinea fowl, pigeon, Eurasian woodcock, and song thrush primers amplified
257 fragments of 129, 113, 141, 130, 133, 138, 110 bp in the 12S rRNA gene,
258 respectively. These small amplicons are ideal for use with processed foods where
259 DNA degradation can mean that larger targets (>150 bp) are not always amplified
260 (Dooley et al. 2004). On the other hand, the endogenous control primer pair was
261 designed to amplify a DNA fragment of 141 bp from all the eukaryotic organisms
262 tested with approximately the same efficiency. All PCR systems used species-
263 specific TaqMan probes, excepting those for partridge, guinea fowl, and pigeon
264 species in which, due to the similar position of the species-specific primers in the 12S
265 rRNA sequence, a common probe was used.

Specificity

Each species-specific PCR system was tested for its selectivity and cross reactivity by analysis of DNAs obtained from different game and domestic meat species. All species were analyzed in duplicate (two separate extractions and PCR reactions from each species).

The quail-specific system amplified a 129 bp fragment from quail (C_p value of 20.39 ± 0.19), whereas no amplification was achieved with DNA from other game and domestic meat species. Similarly, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush-specific systems amplified fragments of 113, 141, 130, 133, 138, and 110 bp, respectively, in samples from each corresponding target species, showing no cross amplification with DNAs from the rest of the meat species analyzed. The C_p values obtained from raw meat samples were 17.1 ± 0.04 in pheasant, 19.14 ± 0.06 in red-legged partridge, 19.23 ± 0.08 in chukar partridge, 19.19 ± 0.10 in barbary partridge, 16.15 ± 0.04 in guinea fowl, 16.32 ± 0.07 in common pigeon, 16.40 ± 0.04 in woodpigeon, 18.22 ± 0.06 in Eurasian woodcock, and 16.03 ± 0.08 in song thrush, whereas in non-template controls no amplification signal was detected after 35 cycles (Table 2). The endogenous control system amplified a 141 bp fragment from all the eukaryotic species analyzed. With this system, all tested species showed C_p values between 16.69 ± 0.03 and 20.30 ± 0.06 .

Efficiency and detection limit

The efficiency and detection limit of the real-time PCR assay developed were evaluated from the standard curve made with the experimental binary mixtures containing 50%, 25%, 10%, 5%, 1%, and 0.1% of either quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, or song thrush meat in a chicken meat matrix. Five ng of DNA from each binary mixture percentage was analyzed with the

corresponding species-specific system and with the endogenous control system. Normalization was carried out on fluorescence results obtained for each mixture with the seven species-specific systems and with the endogenous control, and these data were used as the calibration curve for further statistical studies. Results of the efficiency of the PCR approach for raw experimental binary mixtures are shown in Figure 1 and Figure 2. The slope of log input amount vs. ΔC_p has a value of -3.04 for the quail-specific system (1a), -3.26 for pheasant-specific system (1b), -3.09 for partridge-specific system (1c), -3.11 for guinea fowl-specific system (1d), -3.00 for pigeon-specific system (1e), -3.98 for Eurasian woodcock-specific system (1f), and -3.16 for song thrush-specific system (1g), near to the slope's value of -3.32 corresponding to a 100% PCR efficiency. Similarly, Figure 3 and Figure 4 show the results obtained after analyzing the sterilized experimental mixtures.

The theoretical detection limit for each species-specific PCR system was also calculated from the standard curves obtained with the experimental binary mixtures shown in Figures 1-4. The detection limit for each species-specific system was estimated from the corresponding curve as the DNA amount yielding a C_p value of 35. This value was 8.4×10^{-5} ng in quail, 1.62×10^{-5} ng in pheasant, 2.08×10^{-5} ng in partridge, 4.2×10^{-6} ng in guinea fowl, 1.36×10^{-5} ng in pigeon, 6.7×10^{-5} ng in Eurasian woodcock, and 3.34×10^{-6} ng in song thrush. On the other hand, in sterilized binary mixtures, the detection limits for DNA from quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush specific PCR systems were 1.3×10^{-3} ng, 6.45×10^{-5} ng, 4.08×10^{-4} ng, 3.68×10^{-5} ng, 1.21×10^{-5} ng, 5.38×10^{-4} ng, and 3.2×10^{-5} ng, respectively. It should be noted that although sterilization (121 °C for 20 min) reduces the amount of detectable DNA, positive signals were also observed in thermally treated samples containing small percentages of the target species DNA. It

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is widely reported that the intensity of the heating and pressure of food processing treatments such as sterilization clearly affects DNA fragmentation and can lead to false-negative results (Hird et al. 2006). In the present work, the length of the amplified DNA fragments minimized sensitivity to degraded DNA templates.

It can be deduced from the results obtained that the real-time PCR method developed allows quantification of minute amounts of DNA as long as the reference sample used has received the same treatment than the samples analyzed. So, because of the variations found in the amount and quality of amplifiable DNA in the real-time PCR assay developed, different calibration curves should be used in accordance with the target species, the tissue matrix and the heat treatment applied in order to achieve reliable quantification of target meat content.

Sensitivity and linearity

Normalized C_p values obtained from the raw and heat-treated meat mixtures of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush in chicken, versus the logarithm of the DNA concentrations, were used to test the sensitivity of the real-time PCR method. Figures 1-4 show the discriminating capacity, which is the least difference in logarithm of target DNA concentration in the mixture that the analytical method can discriminate with a significant level. Linearity of the quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush real-time PCR response was also analyzed for each experimental raw and heat treated binary mixture. In each case, the following parameters were evaluated: (a) Cochran's test, which determined whether the variances of the responses obtained for each concentration of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, or song thrush DNA in the mixtures (raw and heat-treated) were

homogeneous, (b) regression analysis, and (c) variance analysis with Lack-of-Fit. The results are also summarized in Figures-4.

Accuracy and precision

The accuracy of the assay was determined from three meat percentages (25%, 10%, and 1%) of each raw and heat-treated binary mixture. Accuracy is reported as percentage recovery by the assay of known added amounts of analyte. Using the Snedecor F-test, it was verified that the concentration of DNA present in a sample did not affect the variation of the results. The values of the Student's t-test obtained demonstrated that the method was accurate, since no significant differences between 100% and the mean recovery values were detected.

The influence of the day of analysis on the precision of the assay was also tested by comparing the coefficients of variation (CV) of three separate DNA extractions performed on the 1% raw and sterilized binary mixtures, analyzed in duplicate three times on the same day, versus the values obtained for the same samples on three different days. Results showed significant differences with higher CV values when the assay was done on different days with respect to the same day. This could be ascribed to the fact that different master mix vials were used in the assays performed on different days and it could be also attributed to minimal volumetric variations caused by pipetting errors in any of the steps of the real-time PCR experiment (DNA aliquot preparation, mix of reagents, dispensation of the master mix into the PCR capillaries, etc.). Thus, for the quantification of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush DNAs, a standard curve should be determined on each day of analysis.

Application of the assay to commercial meat products

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The method was also tested with regard to its suitability for detection of game bird DNA in commercial meat products. For that purpose DNA samples extracted from quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush meat products were analyzed. As can be seen in Tables 3 and 4, the specific and endogenous C_p values obtained from the DNA samples extracted from these commercial products were very variable, including those obtained in different pâté samples with similar specified content of the target species (Table 4). This can be attributed to a variation in factors such as meat quality, tissue type, matrix composition, and levels of processing. It should be noted that, in most cases, the results obtained from the analysis of meat products were in agreement with the declaration of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, or song thrush content on the label. However, seven partridge pâtés and one pheasant pâté, which were supposed to contain partridge and pheasant respectively, did not produce positive results with the corresponding species-specific real-time PCR systems. Nevertheless, successful results were obtained with the endogenous control system, suggesting a possible fraud in the labeling of these pâté samples (Table 4). In addition, commercial meat samples containing more than one species in their composition were also tested for the presence of other animal species stated in the label such as chicken, turkey, cattle, swine and duck. For this purpose, conventional PCR assays using species-specific primers for the mentioned species were carried out. In all the samples tested, the results obtained were in accordance with the species composition stated in the labels including the pâté samples in which partridge and pheasant were not detected (Table 4).

In order to test the usefulness of the method for the quantification of the target species in commercial meat products containing more than one species in their

composition, a quantitative measurement was performed substituting the normalized C_p values obtained for these products in the standard equations derived from the analysis of the experimental sterilized binary mixtures. As can be seen in Table 4 the results obtained indicated a complete disagreement between the amounts detected and the declared content of each target species. Although, as previously described, this is a technique with quantitative potential, the use of the standard equations developed in this work does not turn out to be suitable for the quantification of commercial meat products because of two main reasons: the composition of the commercial meat products that due to the presence of different ingredients could influence in the real-time reaction, and the fact that it was not possible to know if the heat-treatment applied to each product was similar to the sterilization treatment used in the present work (121 °C, 20 min). In consequence, the C_p values measured on commercial meat products can only be used for a quantitative estimate of the target species content if samples identical in composition and processing treatment to the analyzed products are available for the preparation of appropriate calibration standards. Taking into account the great variety of ingredients used in the preparation of commercial meat products (meat, liver, fat, oil, milk, butter, vegetables, etc.) as well as the diversity of heat treatments applied by the meat industry, the preparation of calibration standards for each type of product is not feasible. Therefore, the use of the present real-time PCR technique for the analysis of commercial meat products is limited to qualitative detection of the target species. Nevertheless it should be noted that quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush qualitative detection in commercial meat products was not hindered by the heat and pressure processing of autoclaving, oven

cooking, pickling, or by the presence of other meats, indicating the suitability of the assays for the testing of complex commercial food products (Chisholm et al. 2008).

In conclusion, TaqMan real-time polymerase chain reaction is a powerful technology that is simple and relatively fast, with good sensitivity and specificity. The potential of the described procedure to detect small amounts of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush DNA in raw and heat-treated meats may make it a useful tool for inspection programs to enforce labeling regulations of game bird meat products.

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REFERENCES

1. Camacho MA, Torres AI, Gil-Alegre ME, Obregón MM, Ruz V. 1993. Validation protocol of analytical methods for finished pharmaceutical products. STP Pharm Pract. 3:197-202.
2. Caporale V, Giovannini A, Di Francesco C, Calistri, P. 2001. Importance of the traceability of animals and animal products in epidemiology. Rev Sci Tech Off Int Epiz. 20:372-378.
3. Che Man YB, Aida AA, Raha AR, Son R. 2007. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control. 18:885-889.
4. Chisholm J, Conyers C, Booth C, Lawley W, Hird H. 2005. The detection of horse and donkey using real-time PCR. Meat Sci. 70:727-732.
5. Chisholm J, Sánchez A, Brown J, Hird H. 2008. The development of species-specific real-time PCR assays for the detection of pheasant and quail in food. Food Anal Methods. 1:190-194.
6. Di Pinto A, Forte VT, Conversano MC, Tantillo GM. 2005. Duplex polymerase chain reaction for detection of pork meat in horse meat fresh sausages from Italian retail sources. Food Control. 16:391-394.
7. Dooley JJ, Paine KE, Garrett SD, Brown HM. 2004. Detection of meat species using TaqMan real-time PCR assays. Meat Sci. 68:431-438.
8. Fajardo V, González I, Martín I, López-Calleja I, Rojas M, Hernández PE, García T, Martín R. 2007. PCR identification of meats from chamois (*Rupicapra rupicapra*), pyrenean ibex (*Capra pyrenaica*), and mouflon (*Ovis ammon*)

1
2
3 460 targeting specific sequences from the mitochondrial D-loop region. Meat Sci.
4
5 461 76:644-652.
6
7
8 462 9. Fajardo V, González I, Martín I, Rojas M, Hernández PE, García T, Martín R.
9
10 463 2008a. Real-time PCR for detection and quantification of red deer (*Cervus*
11
12 464 *elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat
13
14 465 mixtures. Meat Sci. 79:289-298.
15
16
17
18 466 10. Girish PS, Anjaneyulu ASR, Viswas KN, Anand M, Rajkumar N, Shivakumar BM,
19
20 467 Sharma B. 2004. Sequence analysis of mitochondrial 12S rRNA gene can identify
21
22 468 meat species. Meat Sci. 66:551-556.
23
24
25
26 469 11. Hird H, Chisholm J, Sánchez A, Hernández M, Goodier R, Schneede K. 2006.
27
28 470 Effect of heat and pressure processing on DNA fragmentation and implications for
29
30 471 the detection of meat using a real-time polymerase chain reaction. Food Addit
31
32 472 Contam. 23:645-650.
33
34
35
36 473 12. ICH Harmonised Tripartite Guideline. 2005. Validation of analytical procedures:
37
38 474 text and methodology Q2 (R1).
39
40
41 475 13. Kesmen Z, Sahin F, Yetim H. 2007. PCR assay for the identification of animal
42
43 476 species in cooked sausages. Meat Sci. 77:649-653.
44
45
46
47 477 14. Martín I, García T, Fajardo V, López-Calleja I, Rojas M, Hernández PE, González
48
49 478 I, Martín R. 2007a. Mitochondrial markers for the detection of four duck species
50
51 479 and the specific identification of Muscovy duck in meat mixtures using the
52
53 480 polymerase chain reaction. Meat Sci. 76:721-729.
54
55
56
57 481 15. Martín I, García T, Fajardo V, López-Calleja I, Hernández PE, González I, Martín
58
59 482 R. 2007b. Species-specific PCR for the identification of ruminant species in
60
483 feedstuffs. Meat Sci. 75:120-127.

16. Martín I, García T, Fajardo V, López-Calleja I, Rojas M, Pavón MA, Hernández PE, González I, Martín R. 2007c. Detection of chicken, turkey, duck, and goose tissues in feedstuffs, using species-specific polymerase chain reaction. J Anim Sci. 85:452-458.
17. Martín I, García T, Fajardo V, Rojas M, Pegels N, Hernández PE, González I, Martín R. 2008. SYBR-Green real-time PCR approach for the detection and quantification of pig DNA in feedstuffs. Meat Sci. 82:252-259.
18. Rodríguez MA, García T, González I, Asensio L, Hernández PE, Martín R. 2004. Quantitation of mule duck in goose foie gras using TaqMan real-time polymerase chain reaction. J Agric Food Chem. 52:1478-1483.
19. Rojas M, González I, Fajardo V, Martín I, Hernández PE, García T, Martín R. 2008. Polymerase chain reaction-restriction fragment length polymorphism authentication of raw meats from game birds. J. AOAC Int. 91:1416-1422.
20. Rojas M, González I, Fajardo V, Martín I, Hernández PE, García T, Martín R. 2009. Authentication of meats from quail (*Coturnix coturnix*), pheasant (*Phasianus colchicus*), partridge (*Alectoris* spp.), and guinea fowl (*Numida meleagris*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. Food Control. 20:896-902.
21. Tanabe S, Hase M, Yano T, Sato M, Fujimura T, Akiyama H. 2007. A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. Biosci Biotechnol Biochem. 71:3131-3135.
22. Teletchea F, Maudet C, Hänni C. 2005. Food and forensic molecular identification: Update and challenges. Trends Biotechnol. 23:359-366.

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507 23.Verbeke W, Ward RW. 2006. Consumer interest in information cues denoting
508 quality, traceability and origin: an application of ordered probit models to beef
509 labels. Food Qual Prefer. 17:453-467.

510 24.Woolfe M, Primrose S. 2004. Food forensics: using DNA technology to combat
511 misdescription and fraud. Trends Biotechnol. 22:222-226.

512 25.Zhang C, Fowler MR, Scott NW, Lawson G, Slater A. 2007. A TaqMan real-time
513 PCR system for the identification and quantification of bovine DNA in meats, milks
514 and cheeses. Food Control. 18:1149-1158.

FIGURE CAPTIONS

FIGURE 1. Normalized C_p values obtained from raw meat mixtures of quail (1a), pheasant (1b), partridge (1c), and guinea fowl (1d) in chicken, plotted versus the logarithm of the DNA concentrations. Linearity test, regression line and sensitivity parameters are calculated for each species-specific PCR system. Three different DNA extractions performed on different percentages of quail, pheasant, partridge, or guinea fowl (50, 25, 10, 5, 1 and 0.1%) species in the raw meat mixtures were used as standards.

FIGURE 2. Normalized C_p values obtained from raw meat mixtures of pigeon (2a), Eurasian woodcock (2b), and song thrush (2c) in chicken, plotted versus the logarithm of the DNA concentrations. Linearity test, regression line and sensitivity parameters are calculated for each species-specific PCR system. Three different DNA extractions performed on different percentages of pigeon, Eurasian woodcock, or song thrush (50, 25, 10, 5, 1 and 0.1%) species in the raw meat mixtures were used as standards.

FIGURE 3. Normalized C_p values obtained from sterilized meat mixtures of quail (3a), pheasant (3b), partridge (3c), and guinea fowl (3d) in chicken, plotted versus the logarithm of the DNA concentrations. Linearity test, regression line and sensitivity parameters are calculated for each species-specific PCR system. Three different DNA extractions performed on different percentages of quail, pheasant, partridge, or guinea fowl (50, 25, 10, 5, 1 and 0.1%) species in the sterilized meat mixtures were used as standards.

FIGURE 4. Normalized C_p values obtained from sterilized meat mixtures of pigeon (4a), Eurasian woodcock (4b), and song thrush (4c) in chicken, plotted versus the

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540 parameters are calculated for each species-specific PCR system. Three different
541 DNA extractions performed on different percentages of pigeon, Eurasian woodcock,
542 or song thrush (50, 25, 10, 5, 1 and 0.1%) species in the sterilized meat mixtures
543 were used as standards.

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FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

TABLE 1

DNA sequences of the primers and probes used in this study

Primers and probes	Length (bp)	Sequence (5' to 3')	Description	Optimal concentration (nM)
18SpEU-DIR	29	GGTAGTGACGAAAAATAACAATACAGGAC	Endogenous control forward primer	300
18SpEU-INV	25	ATACGCTATTGGAGCTGGAATTACC	Endogenous control reverse primer	900
12SCOT-FW	27	GATTAGCAGTAAATGGGATCACTTT	Quail forward primer	300
12SCOT-REV	22	TCGTCCTTGGCTTAATGGTTGG	Quail reverse primer	900
12SPHA-FW	23	AGTGGTCATATGTTATCCTCACC	Pheasant forward primer	900
12SPHA-REV	21	GGGGTAAAAATTAGTCGTGGAG	Pheasant reverse primer	900
12SALEC-FW	26	CGACCTAAAAACCATCTTAGTTCCCA	Partridge forward primer	300
12SALEC-REV	24	CGTAGTTCTCGGGCGGATATATTG	Partridge reverse primer	900
12SNUM-FW	24	ACCTCAAAACAATCTTAGCCACCA	Guinea fowl forward primer	300
12SNUM-REV	25	TTCTCAGGCGGATACTTAGGTATTG	Guinea fowl reverse primer	900
12SCOLU-FW	26	GCCCTAAATACGACCCTAAACTCATA	Pigeon forward primer	900
12SCOLU-REV	23	CAGGCGGATGCTTTGGTTATATA	Pigeon reverse primer	900
12SSCO-FW	27	CAGCTCACCTCCAATGAGAGCTATATA	Eurasian woodcock forward primer	300
12SSCO-REV	21	ATGCCCCCTTCCGTATGGTTA	Eurasian woodcock reverse primer	300
12STUR-FW	24	GAGTGGTCGCATGTTATCCAAGTA	Song thrush forward primer	300
12STUR-REV	29	AATCGTTGTTCTAAGATCTTCTTGAAGA	Song thrush reverse primer	900
18SPROBE	28	FAM-AAGTGGA CTATTCCAATTACAGGGCCXT-TMR	Endogenous control probe	200
QUAI-TM	24	FAM-CTTCAAAAGCTACTAATACCGATA-TMR	Quail probe	200
PHEASANT-TM	30	FAM-CCTAAGATCGAAGTACAACCTAAGCTGTCAT-TMR	Pheasant probe	200
BIRDS-TM	25	FAM-ACGAAAGCCAGGRCACAACTGGGA-TMR	Partridge, G. fowl and Pigeon probe	200
WOODCOCK-TM	29	FAM-CACAACCCACTAACAAGACAGGTCGAGGT-BBQ	Eurasian woodcock probe	200
THRUSH-TM	29	FAM-TGAGCTGTCATAAGCCCAAGATGCYCATA-BBQ	Song thrush probe	200

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TABLE 2

Specificity of quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock, and song thrush real-time PCR systems (C_p values obtained from 5 ng DNA)

Species	Quail specific PCR system	Pheasant specific PCR system	Partridge specific PCR system	Guinea fowl specific PCR system	Pigeon specific PCR system	E.woodcock specific PCR system	S.thrush specific PCR system
Quail (<i>Coturnix coturnix</i>)	20.39 ± 0.19	35	35	35	35	35	35
Pheasant (<i>Phasianus colchicus</i>)	35	17.01 ± 0.04	35	35	35	35	35
Red-legged partridge (<i>Alectoris rufa</i>)	35	35	19.14 ± 0.06	35	35	35	35
Chukar partridge (<i>Alectoris chukar</i>)	35	35	19.23 ± 0.08	35	35	35	35
Barbary partridge (<i>Alectoris barbara</i>)	35	35	19.19 ± 0.10	35	35	35	35
Guinea fowl (<i>Numida meleagris</i>)	35	35	35	16.15 ± 0.04	35	35	35
Common pigeon (<i>Columba livia</i>)	35	35	35	35	16.32 ± 0.07	35	35
Woodpigeon (<i>Columba palumbus</i>)	35	35	35	35	16.40 ± 0.04	35	35
Eurasian woodcock (<i>Scolopax rusticola</i>)	35	35	35	35	35	18.22 ± 0.06	35
Song thrush (<i>Turdus philomelas</i>)	35	35	35	35	35	35	16.03 ± 0.08
Capercaillie (<i>Tetrao urogallus</i>)	35	35	35	35	35	35	35
Chicken (<i>Gallus gallus</i>)	35	35	35	35	35	35	35
Turkey (<i>Meleagris gallopavo</i>)	35	35	35	35	35	35	35
Muscovy duck (<i>Cairina moschata</i>)	35	35	35	35	35	35	35
Goose (<i>Anser anser</i>)	35	35	35	35	35	35	35
Cattle (<i>Bos taurus</i>)	35	35	35	35	35	35	35
Sheep (<i>Ovis aries</i>)	35	35	35	35	35	35	35
Goat (<i>Capra hircus</i>)	35	35	35	35	35	35	35
Swine (<i>Sus scrofa dom.</i>)	35	35	35	35	35	35	35
Fallow deer (<i>Dama dama</i>)	35	35	35	35	35	35	35
Roe deer (<i>Capreolus capreolus</i>)	35	35	35	35	35	35	35
Red deer (<i>Cervus elaphus</i>)	35	35	35	35	35	35	35
Chamois (<i>Rupicapra rupicapra</i>)	35	35	35	35	35	35	35
Mouflon (<i>Ovis ammon</i>)	35	35	35	35	35	35	35
Pyrenean ibex (<i>Capra pyrenaica</i>)	35	35	35	35	35	35	35

Average C_p value ± SD shown from duplicate reactions
C_p of 35 indicates no amplification after 35 cycles

TABLE 3

C_p values obtained for PCR products amplified with species-specific primers from DNA extracted of raw and heat-treated commercial meat products containing only one species in their composition. (C_p values produced using 5 ng DNA).

Type of product	Quail specific PCR system	Pheasant specific PCR system	Partridge specific PCR system	Guinea fowl specific PCR system	Pigeon specific PCR system	E. woodcock specific PCR system	Song thrush specific PCR system	Endogenous control PCR system
Quail pickled thighs A	23.81 ± 0.13	35	35	35	35	35	35	23.14 ± 0.13
Quail pickled thighs B	23.14 ± 0.08	35	35	35	35	35	35	21.56 ± 0.08
Quail pickled meat	24.43 ± 0.09	35	35	35	35	35	35	20.52 ± 0.13
Quail boned pickled meat	20.94 ± 0.18	35	35	35	35	35	35	21.48 ± 0.18
Quail braised meat	20.80 ± 0.21	35	35	35	35	35	35	20.27 ± 0.21
Pheasant braised meat A	35	23.52 ± 0.08	35	35	35	35	35	22.38 ± 0.14
Pheasant braised meat B	35	21.86 ± 0.11	35	35	35	35	35	22.07 ± 0.21
Pheasant ragout	35	24.45 ± 0.23	35	35	35	35	35	23.19 ± 0.13
Partridge pickled thighs A	35	35	24.66 ± 0.27	35	35	35	35	22.48 ± 0.26
Partridge pickled meat A	35	35	20.61 ± 0.21	35	35	35	35	23.50 ± 0.22
Partridge pickled meat B	35	35	25.89 ± 0.14	35	35	35	35	24.04 ± 0.17
Partridge pickled meat C	35	35	28.54 ± 0.07	35	35	35	35	25.02 ± 0.13
Partridge pickled meat D	35	35	24.58 ± 0.21	35	35	35	35	24.28 ± 0.18
Partridge pickled meat E	35	35	27.85 ± 0.11	35	35	35	35	25.30 ± 0.21
Partridge pickled meat F	35	35	25.22 ± 0.16	35	35	35	35	23.25 ± 0.22
Partridge boned pickled meat A	35	35	28.61 ± 0.06	35	35	35	35	25.34 ± 0.17
Partridge boned pickled meat B	35	35	27.36 ± 0.21	35	35	35	35	24.35 ± 0.11
Partridge stewed meat	35	35	19.67 ± 0.11	35	35	35	35	18.02 ± 0.23
Partridge braised meat A	35	35	24.22 ± 0.13	35	35	35	35	22.35 ± 0.17
Partridge braised meat B	35	35	25.72 ± 0.16	35	35	35	35	24.74 ± 0.17
G. fowl braised meat A	35	35	35	19.09 ± 0.17	35	35	35	19.48 ± 0.19
G. fowl braised meat B	35	35	35	19.97 ± 0.24	35	35	35	20.14 ± 0.24
G. fowl stewed meat	35	35	35	21.48 ± 0.11	35	35	35	22.85 ± 0.17
Pigeon pickled thighs	35	35	35	35	26.13 ± 0.32	35	35	24.76 ± 0.18
Pigeon pickled meat A	35	35	35	35	25.80 ± 0.15	35	35	22.29 ± 0.12
Pigeon pickled meat B	35	35	35	35	24.87 ± 0.05	35	35	24.14 ± 0.37
Pigeon braised meat	35	35	35	35	26.43 ± 0.22	35	35	20.76 ± 0.23
E. woodcock refrigerated meat A	35	35	35	35	35	18.25 ± 0.32	35	21.85 ± 0.14
E. woodcock refrigerated meat B	35	35	35	35	35	18.06 ± 0.12	35	21.94 ± 0.05
E. woodcock frozen meat	35	35	35	35	35	18.41 ± 0.14	35	22.09 ± 0.28
S. thrush refrigerated meat A	35	35	35	35	35	35	16.03 ± 0.25	19.54 ± 0.16
S. thrush refrigerated meat B	35	35	35	35	35	35	15.81 ± 0.08	19.40 ± 0.11

Average C_p value ± SD shown from duplicate reactions
 C_p of 35 indicates no amplification.

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TABLE 4

C_p values obtained for PCR products amplified with species-specific primers from DNA extracted of commercial meat products containing more than one species in their composition. (C_p values produced using 5 ng DNA).

Type of product	Quail specific PCR system	Pheasant specific PCR system	Partridge specific PCR system	Guinea fowl specific PCR system	Pigeon specific PCR system	Endogenous control PCR system	Declared content of the target species	Estimated content of the target species	Other species detected according to the label description
Quail pâté A	26.56 ± 0.07	35	35	35	35	25.01 ± 0.07	40%	79%	Cattle, swine, chicken, duck
Quail pâté B	23.37 ± 0.17	35	35	35	35	22.11 ± 0.07	30%	85%	Cattle, swine, chicken
Quail paté C	25.08 ± 0.12	35	35	35	35	20.77 ± 0.17	11%	8%	Cattle, swine, chicken, turkey
Quail paté C	21.62 ± 0.15	35	35	35	35	20.23 ± 0.22	30%	71%	Cattle, swine, chicken
Pheasant pâté A	35	19.75 ± 0.08	35	35	35	18.62 ± 0.27	20%	4%	Cattle, swine, chicken
Pheasant pâté B	35	21.48 ± 0.03	35	35	35	20.73 ± 0.18	25%	5.5%	Swine, turkey
Pheasant pâté C	35	22.63 ± 0.08	35	35	35	24.07 ± 0.11	25%	24%	Cattle, swine, chicken, turkey
Pheasant pâté D	35	24.98 ± 0.23	35	35	35	23.07 ± 0.11	25%	3%	Cattle, swine, chicken, duck
Pheasant pâté E	35	21.45 ± 0.12	35	35	35	21.06 ± 0.12	25%	7%	Cattle, swine, chicken, turkey
Pheasant pâté F	35	35	35	35	35	23.21 ± 0.24	32%	Not detected	Cattle, swine, chicken, turkey
Pheasant pâté G	35	28.01 ± 0.15	35	35	35	22.34 ± 0.12	2%	0.2%	Cattle, swine, chicken
Pheasant pâté H	35	21.66 ± 0.14	35	35	35	21.35 ± 0.09	25%	8%	Cattle, swine, chicken, turkey
Partridge pâté A	35	35	35	35	35	22.05 ± 0.08	30.5%	Not detected	Cattle, swine, chicken
Partridge paté B	35	35	27.24 ± 0.23	35	35	21.66 ± 0.21	30%	8%	Cattle, swine, duck
Partridge pâté C	35	35	23.55 ± 0.22	35	35	19.58 ± 0.28	- ^a	17%	Cattle, swine
Partridge pâté D	35	35	26.61 ± 0.09	35	35	21.32 ± 0.27	-	9%	Cattle, swine, chicken, turkey
Partridge pâté E	35	35	35	35	35	20.45 ± 0.24	20%	Not detected	Cattle, swine, chicken, duck
Partridge pâté F	35	35	35	35	35	20.68 ± 0.32	20%	Not detected	Cattle, swine, chicken, duck
Partridge pâté G	35	35	24.15 ± 0.09	35	35	21.32 ± 0.07	-	45%	Cattle, swine, chicken, turkey
Partridge pâté H	35	35	26.90 ± 0.12	35	35	18.05 ± 0.15	-	0.3%	Cattle, swine, chicken, duck
Partridge pâté I	35	35	28.34 ± 0.23	35	35	21.55 ± 0.11	-	3.5%	Cattle, swine, chicken
Partridge pâté J	35	35	35	35	35	21.31 ± 0.22	22%	Not detected	Cattle, swine, chicken
Partridge pâté K	35	35	35	35	35	20.45 ± 0.25	25%	Not detected	Cattle, swine, chicken, duck

TABLE 4 (Continued)

Type of product	Quail specific PCR system	Pheasant specific PCR system	Partridge specific PCR system	Guinea fowl specific PCR system	Pigeon specific PCR system	Endogenous control PCR system	Declared content of the target species	Estimated content of the target species	Other species detected according to the label description
Partridge pâté L	35	35	28.62 ± 0.24	35	35	21.93 ± 0.13	-	4%	Cattle, swine, chicken, turkey
Partridge pâté M	35	35	24.31 ± 0.13	35	35	21.91 ± 0.31	25%	62%	Cattle, swine, chicken, duck
Partridge pâté N	35	35	35	35	35	20.11 ± 0.21	22%	Not detected	Cattle, swine, chicken
Partridge pâté O	35	35	35	35	35	21.87 ± 0.08	30.5%	Not detected	Cattle, swine, chicken
G. fowl pâté A	35	35	35	24.42 ± 0.21	35	23.14 ± 0.14	34%	1%	Swine, chicken, turkey
G. fowl pâté B	35	35	35	25.52 ± 0.08	35	25.56 ± 0.05	20%	2.5%	Swine, duck
G. fowl pâté C	35	35	35	25.12 ± 0.16	35	24.61 ± 0.05	20%	2%	Cattle, chicken, turkey
G. fowl pâté D	35	35	35	22.62 ± 0.23	35	24.02 ± 0.21	34%	7%	Swine, chicken, turkey
Pigeon pâté A	35	35	35	35	20.27 ± 0.13	23.51 ± 0.29	10%	26%	Cattle, swine, chicken, turkey, duck
Pigeon pâté B	35	35	35	35	20.45 ± 0.21	23.08 ± 0.15	10%	17%	Cattle, swine, chicken, turkey, duck
Pigeon pâté C	35	35	35	35	22.97 ± 0.14	23.20 ± 0.24	-	3%	None
Pigeon pâté D	35	35	35	35	23.24 ± 0.16	22.98 ± 0.12	-	2%	None

Average C_p value ± SD shown from duplicate reactions

C_p of 35 indicates no amplification.

^a means not specified content of the target species