Analysis of pork adulteration in commercial meatballs targeting porcine-specific mitochondrial cytochrome b gene by TaqMan probe real-time polymerase chain reaction

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Abstract

A test for assessing pork adulteration in meatballs, using TaqMan probe real-time polymerase chain reaction, was developed. The assay combined porcine-specific primers and TaqMan probe for the detection of a 109 bp fragment of porcine cytochrome b gene. Specificity test with 10 ng DNA of eleven different species yielded a threshold cycle (Ct) of 15.5±0.20 for the pork and negative results for the others. Analysis of beef meatballs with spiked pork showed the assay can determine 100–0.01% contaminated pork with 102% PCR efficiency, high linear regression (r²=0.994) and ≤6% relative errors. Residuals analysis revealed a high precision in all determinations. Random analysis of commercial meatballs from pork, beef, chicken, mutton and goat, yielded a Ct between 15.89±0.16 and 16.37±0.22 from pork meatballs and negative results from the others, showing the suitability of the assay to determine pork in commercial meatballs with a high accuracy and precision.

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A R T I C L E  A B S T R A C T

Introduction

Transparency in meat speciation is an ever increasing demand and is essential for the protection of consumers’ heath, religious credence and hard earned wealth (Ali, Hashim, Mustafa, Che Man, Yusop, Bari, et al., 2011; Ali, Hashim, Mustafa, Che Man, Yusop, Kashif, et al., 2011). Consumers must know accurate information of the product materials before they make any purchase decision. EC legislation (178/2002) on food safety (European Commission, 2002) clearly defines that each stake holder in a food supply chain must be able to trace out all raw materials utilized in the manufacturing of food products. As minced meats are being increasingly used in modern food, a convenient method of verifying labeling of commercial food products is of great value to ensure food safety and gain consumers’ trust (Ali, Hashim, Mustafa, & Che Man, 2011). Meatballs made with comminuted meat are popular throughout the world. They can be formulated using beef, chicken, pork, and/or fish muscle. However, beef meatball is very popular and widely found in markets (Rahman, Sismindy, Erwanto, & Che Man, 2011). Substitution of beef in meatball formulations with lower valued meats such as pork frequently takes place to coup up with market competition and also to earn economic gain. The presence of pork or any of its derivatives in food products is a serious religious concern as pork is banned by the religious laws of Islam and Judaism (Ali, Hashim, Mustafa, & Che Man, 2011).

A number of analytical methods have been proposed for the analysis of pork and/or lard, such as electronic nose coupled to gas chromatography–mass spectrometry (Nurjuliana, Che Man, Mat Hashim, & Mohamed, 2011), Fourier transform infrared spectroscopy (Rahman et al., 2011), enzyme-linked immunosorbanant assay (Asensio, González, García, & Martín, 2008), PCR-electrophoresis (Che Man, Mustafa, Khaliril Mokhtar, Nordin, & Sazili, 2012), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2011), TaqMan probe real-time PCR (Köppel, Ruf, & Rentsch, 2011), molecular beacon real-time PCR (Yusop, Mustafa, Che Man, Omar, & Moktar, 2011), SYBR green real-time PCR (Farrukhi & Jafari Joozani, 2011), and nanoparticle sensors coupled with optical or fluorescence spectroscopy (Ali, Hashim, Mustafa, Che Man, & Islam, 2012; Ali, Hashim, Mustafa, Che Man, Yusop, Bari, et al., 2011; Ali, Hashim, Mustafa, Che Man, Yusop, Kashif, et al., 2011). Among these methods, real-time PCR assays combined with species-specific primers and TaqMan probe are particularly promising because in addition to specific primers, additional species-screening is provided through the specifically-designed TaqMan probe, significantly enhancing the specificity and reliability of the assay (Köppel et al., 2011; Sakai et al., 2011). Both the simple...
(Rojas et al., 2010) and multiplex (Köppel et al., 2011) real-time PCR assays with TaqMan probes are proposed. Multiplex PCR assays allow simultaneous identification of several species using a single-PCR assay, reducing both cost and time (Köppel et al., 2011). However, they use comparatively longer and variable-length DNA templates (Köppel et al., 2011; Sakai et al., 2011) which are not stable in the harsh conditions of food-processing (Bielikova, Pangallo, & Turna, 2010) and also entails different sensitivities for different species, making them unsuitable for the analysis of processed commercial goods (Ali, Hashim, Mustafa, & Che Man, 2011). Additionally, an optimized real-time PCR assay under a background of complex matrices found in processed commercial foods has yet to be described.

Thus, the development of an optimized real-time PCR assay in a background of heterogeneous food components with shorter amplicon-length is of great value as shorter amplicons are less affected by degradation and shows better recovery of target sequences even in compromised samples (Ali, Hashim, Mustafa, & Che Man, 2011). In this paper, we combined the species-specific primers and TaqMan probe to specifically amplify and detect a short fragment (109-bp) of porcine mitochondrial (mt) cytb gene by real-time PCR. A 31-nt and a 28-nt TaqMan hydrolysis probes specific for the porcine and endogenous PCR systems with double quenchers, ZEN probe (Integrated DNA technologies (IDT), USA) in the middle and Iowa Black at the end, were used to enhance interspecies polymorphism and intraspecies specificity. The accuracy and precision of the method was tested in model experiments, validated in ready-to-eat beef meatballs with spiked pork and justified in commercially sourced 45 meatballs of 5 meat species. The assay was further authenticated by RFLP analysis.

2. Materials and methods

2.1. Sample collections

The fresh muscle of 6 meat-producing terrestrial species such as pig (Sus scrofa), cow (Bos taurus), sheep (Ovis aries), goat (Capra hircus), deer (Cervus nippon), and chicken (Gallus gallus) and 5 aquatic species such as chichlid (Crenicichla minuana), shad (Alosa sapidissima), shrimp (Gadus morhua), tuna (Thunnus orientalis), and cuttlefish (Sepia officinalis) animal species were purchased in triplicates on three different days from Pasar Borong, Selangor, Malaysia. The identity of the source animals was verified by the veterinary and fishery experts from the Department of Animal Sciences in the Universiti Putra Malaysia. The samples were cut into small pieces and stored frozen at −20 °C until use to prevent enzymatic degradation of DNA.

Commercial meatballs of pork, beef, chicken, mutton and chevon/goat of three different local brands (Tip Top Meat Sdn. Bhd., Mariam Goods (Ali, Hashim, Mustafa, & Che Man, 2011). Additionally, an opti-

2.2. Preparation of meatballs

Meatballs were prepared according to Rahman et al. (2011) by mixing 90% ground meat with 10% tapioca starch, cooking salt, garlic and other spices. The mixture was emulsified and mechanically given to a ball shape. The prepared meatballs were cooked in boiling water for 15 min to make it suitable for eating.

2.3. Calibration and validation standards

To prepare the calibration set of beef meatballs, minced pork was spiked with ground beef to make 0.01, 0.1, 1.0 and 10% (w/w) pork contamination and was mixed with 10% tapioca starch, cooking salt, garlic and other spices. The mixture was emulsified and mechanically given to a ball shape. Meatballs containing 100% beef and 100% pork were also made. A validation set of similar composition was prepared and randomly numbered to eliminate analyst biasedness. All meatballs were cooked in boiling water for 15 min prior to DNA extraction.

2.4. DNA extraction

Total DNA extraction was carried out from 5 ml of muscle tissue of each species using MasterPure™ DNA Purification Kit (Epicenter Biotechnologies, Madison, USA) as per the protocol supplied with the reagent. For commercial samples and formulated meatballs, 1 ml of cell and tissue lysis solution (Epicenter Biotechnologies, Madison, USA) was added to a 100 mg portion of finely chopped sample and was incubated in a shaking water bath at 65 °C for 12 h. The subsequent steps of the extraction protocol were performed according to the Epicenter Biotechnologies. The purity and concentration of extracted DNA samples were determined based on absorbance at A520/A280.

2.5. Primer and probe design

A pair of primers (SwcytbF= TCC TGC CCT GAG GAC AAA TA and SwcytbR = AAG CCC CCT CAG ATT CAT TC) targeting 109-bp fragment of porcine cytb gene was designed by primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). A 31-nt porcine TaqMan probe (SwcytbTqM = 6-FAM/AGC TAC GGT/ZEN/CAT CAC AAA TCT ACT ATC GCT/3IABkFQ) containing a 27-nt Alul-cut fragment within it was designed by tagging 6-carboxyfluorescine (6-FAM) and 3-Iowa black FQ (3-IABkFQ) at the 5’ and 3’-ends. For endogenous control, eukaryotic 18SrRNA specific primers (Eu18SrRNAF and Eu18SrRNAR) and TaqMan probe (Eu18SrRNAQ) described by Rojas et al. (2010) were used. To increase the signal-to-noise ratio, a second quencher known as ZEN probe (IDT) was introduced at the 10th position of both TaqMan probes. All the probes and the primers were purchased from the Integrated DNA Technologies (IDT, USA).

2.6. Real-time PCR analysis

Real-time PCR analysis was carried out in an Eppendorf Mastercycler ep-realexp machine (Eppendorf, Germany) with 20 μl reaction mixture consisting of 1 × SsoFast probe supermix (Bio-Rad, USA), 300 nM of each primer, 200 nM of TaqMan probe and 10 ng of total DNA. Required dilution was performed using sterile deionized water and separate tubes were used for the endogenous control and porcine-specific assays. A two-step amplification program was pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 61 °C for 20 s. Each sample and endogenous control was run in triplicates on three different days by three independent analysts.

2.7. Construction of standard curve and target quantification

A standard curve was constructed by plotting Ct values against the logarithmic concentration of starting porcine DNA in the calibration set of different beef meatballs. The amount of unknown set was quantified by interpolating the Ct values in the standard curve (Rojas et al., 2010).

2.8. Product authentication by RFLP analysis

To verify authentic PCR products of porcine and endogenous PCR systems, RFLP analysis of both PCR products were performed. The porcine and endogenous PCR products were mixed in 1:1 (v/v) ratio and 15 μl products were digested with Alul in a 25 μl reaction mixture containing 2-units of enzyme and 1 × digestion buffer supplied with the enzyme (New England Biolab, USA) for 1 h at 37 °C in a shaking water bath. The digestion was stopped by heating the mixture at 65 °C for 15 min. One microliter (1 μl) of unpurified digestion mixture
was applied into each sample well of Agilent DNA chip and separation was done in 30 min by Agilent 2100 Bioanalyzer (Ali, Hashim, Mustafa, & Che Man, 2011).

3. Results and discussions

3.1. Real-time PCR system

The primary objective of the study was to develop a shorter-size PCR marker for the rapid screening of pork adulterants in the processed Halal and Kosher foods. Shorter-size amplicon offers several advantages over the longer one: (1) less affected by degradation and ensures higher recovery of targets in compromised samples, (2) amplify more efficiently, and (3) can be separated more easily by capillary electrophoresis (Ali, Hashim, Mustafa, & Che Man, 2011). As a target for real-time PCR, a 109-bp fragment of porcine cytb gene was chosen because of its multiple copy number, high rate of evolution, polymorphic features between intra and interspecies and additional protection by mt-membrane (Ali, Hashim, Mustafa, & Che Man, 2011). The designed primers and TaqMan probe were verified by NCBI blast analysis and ClustalW alignment analysis to ensure porcine specificity. A comparatively longer TaqMan probe (31-nt) was used in the present work to ensure enough polymorphism and higher annealing temperature. Higher annealing temperature reduces non-specific amplification and thus, increases specificity (Yusop et al., 2011). To reduce the intra-molecular distance between the 5’-fluorophore (FAM) and 3’-quencher (Iowa black), a second quencher (ZEN-probe) was introduced at the 10th position. A eukaryotic primer set and TaqMan probe that specifically amplify a conserved 141-bp fragment of 18S rRNA was selected as an endogenous control (Rojas et al., 2010).

Since shorter amplicon in PCR assays often compromises specificity (Ali, Hashim, Mustafa, & Che Man, 2011), RFPL analysis was performed to authenticate genuine PCR product. In-silico digestion by NEBcutter V2.0 (New England Biolabs) demonstrated two Alu-sites with potential fragment length of 27, 33 and 49-bp in porcine PCR product (109-bp) and 14-bp and 127-bp in endogenous PCR product (141-bp).

3.2. Specificity test

The specificity of the porcine-specific TaqMan real-time PCR assay was assessed with 10 ng of DNA extracted from the fresh muscle tissues of 6 meat-producing terrestrial (cow, chicken, sheep, goat, deer and pig) and 5 aquatic (shrimp, tuna, cuttlefish, ciclid and shad) animal species, changing sources and analysts on three different days in triplicates. Positive control was amplified in different tubes using a similar experimental set up by changing the primers and probe to evaluate the quality of total amplifiable eukaryotic DNA in each tube (Rojas et al., 2010). The amplification profiles of the porcine-specific PCR system yielded a Ct of 15.3 ± 0.12 to 15.65 ± 0.16 from porcine DNA and background fluorescence from those of other species in a 40 cycle PCR assay, demonstrating the specificity of the assay for porcine cytb gene. Alignment analysis of the porcine-specific primers and probe demonstrated 100% matching only with S. scrofa cytb gene (AF034253.1) and all other species, such as B. taurus (EU807948.1), C. gallus (EU395454.1), O. aries (EU365990.1), C. hircus (EU130780.1), C. nippon (EF139156.1), G. marhua (AM489716.1), T. orientalis (AM498973.1), S. officinalis (EF423081.1), C. mininnuo (GQ139921.1), and A. sapidissima (EU552616.1), revealed huge number of mismatches in the probe and primer binding regions. The endogenous control showed two distinct spectrums of Ct values, centering the terrestrial and aquatic animal species. The means of endogenous Ct (average of 3 determinations) of terrestrial species were between 18.34 ± 0.18 and 19.55 ± 0.14 and those of aquatic species were 28.2 ± 0.11 and 29.15 ± 0.09. ClustalW alignment analysis of the 18S rRNA genes of the above species revealed 100% matching of the primers and probe with the terrestrial species. While the endogenous primers showed complete matching, the probe alignment demonstrated 2–4 nucleotide-mismatching with those of aquatic species. Smith, Vigilant, and Morin (2002) observed mismatches in the probe binding region significantly increase the Ct values, greatly reducing the efficiency of real-time PCR system.

3.3. PCR efficiency and detection limit

Usually, 10-fold serial dilutions of pure DNA are used to construct a standard curve in order to find the detection limit (DL) and efficiency of a PCR system (Yusop et al., 2011). Binary mixtures of two different DNA-species are also utilized to construct a standard curve and find the DL (Rojas et al., 2010). However, they do not reflect the effects of complex matrices often found in processed foods (Ali, Hashim, Mustafa, & Che Man, 2011) and consequently, cannot accurately quantify targets in commercial products (Rojas et al., 2010). To eliminate the above limitations, beef meatballs with various percentages of spiked pork were prepared simulating the common recipe of a commercial meatball (Rahman et al., 2011). As the number of mitochondria and mitochondrial genes are tissue dependent, finely chopped deboned meats of different tissue-types (95% skeletal muscle, 3% liver, 1% intestine, 0.5% heart, and 0.5% kidney) were used for the preparation of meatball meats.

The amplification profiles of 10 ng DNA extracted from beef meatballs with 100%, 10%, 1%, 0.1%, 0.01% and 0% of spiked pork produced strong fluorescence signals from all porcine DNA containing tubes with a mean Ct of 14.91 ± 0.29 for 100% pork to 28.62 ± 0.16 for 0.01% pork, depending on the level of spiked-pork in respective meatball formulations. The endogenous control demonstrated a constant level of amplification with a mean Ct of 19.86 ± 0.11 to 19.5 ± 0.21, showing no significant dependency of endogenous Ct on the level of porcine adulterant. ANOVA test also did not revealed any significant difference in endogenous Ct values at P ≤ 0.05. This was acceptable because the endogenous control was a target of conserved region in eukaryotic 18S rRNA gene which is identical both in S. scrofa (AM711871.1) and B. taurus (AM711877.1) species.

Usually, Ct value of a particular system is needed to be normalized against the endogenous target of a real-time PCR system (Rojas et al., 2010). However, validation experiment with a total of 45 replicates (3 replicates on 3 different days for 5 different meatballs) with different level (0.01–100%) of spiked pork demonstrated no significant differences in endogenous Ct at P ≤ 0.05. This eliminated the need of Ct normalization for the construction of standard curve. Thus, the raw Cts of porcine-specific PCR system obtained from the 9 replicates of each meatball formulation were plotted against the logarithmic value of porcine DNA in each formulation. A good linear regression was obtained with a high correlation coefficient (r² = 0.994) and a slope of −3.271 (Fig. 1). A calculation of PCR efficiency (E) using the formula, E = 10^[−1/slope] − 1, yielded an efficiency of 102% (Fajardo et al., 2008) which was within the recommended range (90–110%) of real-time PCR efficiency (Yusop et al., 2011). Rodriguez et al. (2005) obtained a PCR efficiency of 64.8% and 68.9% in raw and autoclaved pork-beef binary admixture. They used a 411-bp fragment of porcine 12S rRNA gene as amplicon and realized a DL of 0.1% pork in pork-beef binary mixtures. Using a 119-bp ampiclon and molecular beacon probe of porcine cytb gene, Yusop et al. (2011) realized 96% efficiency with pure DNA and 0.1% DL in binary meat-mixtures. However, they have not tested their PCR system in a complex background of processed meat products which often contain different ingredients and also are subjected to different level of degradative treatments, such as heating, pressuring etc. To calculate DL, they considered a Ct > 35 which is not a reliable Ct in real-time PCR (Rojas et al., 2010). The superiority of the present assay over those of Yusop et al. (2011); Rodriguez et al. (2005) and Rojas et al. (2010) is easily
comprehendible since it thoroughly considered the contributions from a complex background of different ingredients and tissue-composition of a typical meatball meat. A very high DL of 0.01% (w/w) pork with Ct of 30 and PCR efficiency of 102% were obtained. The higher PCR efficiency and DL of the present work can be partly attributed to the smaller size-DNA target (109-bp) (Bielikova et al., 2010) and double-quenched TaqMan probe that generated high signal-to-noise ratio in PCR amplification process.

3.4. Validation and recovery

A total of 15 artificial beef meatballs with 0.01–100% of spiked pork, each in triplicates, were randomly selected and analyzed by real-time PCR. The actual values and the determined values are shown in Table 1. A good recovery, ranging from 94% to 106%, was obtained from all determinations. Thus, the relative error of ≤6% was obtained, showing the high accuracy and precision of the developed PCR-method.

3.5. Residuals analysis

Residuals are estimates of experimental error obtained by subtracting the observed responses from the predicted ones. They are regarded as elements of variation unexplained by a chosen model (Nevado, Flores, & Penalvo, 1997). A plot of concentration residuals against fitted Ct showed a random distribution of all concentration variables within ±0.75 of the zero line (Fig. 2). This indicated a high precision and accuracy of the developed PCR method throughout the measured concentrations range (0.001–10 ng of porcine DNA).

3.6. Analysis of commercial meatballs

We evaluated the suitability of the method for the screening of pork adulteration in the meatball formulations of different species. To fulfill the targets, we randomly amplified 10 ng DNA extracted from the meatballs of pork, beef, chicken, mutton and chevon. We collected meatballs from three different manufacturers on three different days from three different selling-spots to evaluate the effects of variation in cooking methods as well as the quality and sources of meats species used in each preparation. The results are summarized in Table 2. Table 2 shows only porcine-meatball DNA amplified with a mean Ct from 15.89 ± 0.16 to 16.37 ± 0.22 with the porcine-specific real-time PCR system. ANOVA test revealed no significant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Actual (ng)</th>
<th>Found (ng)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10.12</td>
<td>101.2</td>
</tr>
<tr>
<td>2</td>
<td>0.001</td>
<td>0.00094</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.1055</td>
<td>105.5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>9.74</td>
<td>97.4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1.038</td>
<td>103.8</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1.06</td>
<td>106</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>0.0956</td>
<td>95.6</td>
</tr>
<tr>
<td>8</td>
<td>0.001</td>
<td>0.00106</td>
<td>106</td>
</tr>
<tr>
<td>9</td>
<td>0.01</td>
<td>0.097</td>
<td>97</td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td>0.0104</td>
<td>104</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>9.906</td>
<td>99.6</td>
</tr>
<tr>
<td>12</td>
<td>0.01</td>
<td>0.0102</td>
<td>102</td>
</tr>
<tr>
<td>13</td>
<td>0.1</td>
<td>0.0955</td>
<td>95.5</td>
</tr>
<tr>
<td>14</td>
<td>0.001</td>
<td>0.00103</td>
<td>103</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>9.896</td>
<td>98.96</td>
</tr>
</tbody>
</table>

Table 1
Comparison of actual values with real-time PCR results for the determination of pork in beef meatballs (average of three determinations).

Table 2
Ct values obtained from commercial meatballs with pork-specific and endogenous real-time PCR systems.

<table>
<thead>
<tr>
<th>Meatball</th>
<th>Mean Ct</th>
<th>Number of positive replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>15.89 ± 0.16a</td>
<td>19.5 ± 0.18b</td>
</tr>
<tr>
<td>Day 2</td>
<td>16.37 ± 0.22a</td>
<td>19.17 ± 0.18b</td>
</tr>
<tr>
<td>Day 3</td>
<td>15.92 ± 0.30a</td>
<td>19.46 ± 0.09b</td>
</tr>
<tr>
<td>Beef</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>40b</td>
<td>19.97 ± 0.23a</td>
</tr>
<tr>
<td>Day 2</td>
<td>40b</td>
<td>19.63 ± 0.20a</td>
</tr>
<tr>
<td>Day 3</td>
<td>40b</td>
<td>19.49 ± 0.17a</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>40b</td>
<td>19.44 ± 0.20e</td>
</tr>
<tr>
<td>Day 2</td>
<td>40b</td>
<td>19.02 ± 0.15e</td>
</tr>
<tr>
<td>Day 3</td>
<td>40b</td>
<td>19.11 ± 0.16e</td>
</tr>
<tr>
<td>Mutton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>40b</td>
<td>19.51 ± 0.24c</td>
</tr>
<tr>
<td>Day 2</td>
<td>40b</td>
<td>19.17 ± 0.20c</td>
</tr>
<tr>
<td>Day 3</td>
<td>40b</td>
<td>19.09 ± 0.23c</td>
</tr>
<tr>
<td>Chevon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>40b</td>
<td>19.56 ± 0.16d</td>
</tr>
<tr>
<td>Day 2</td>
<td>40b</td>
<td>19.27 ± 0.17d</td>
</tr>
<tr>
<td>Day 3</td>
<td>40b</td>
<td>18.49 ± 0.17f</td>
</tr>
</tbody>
</table>

Mean Cts within the same columns with the same letter are not significantly different at P≤0.05 as determined by Turkey’s family error test in one way ANOVA.
difference at $P \leq 0.05$ with a change of analyst and sources, reflecting its suitability to screen commercial meatballs. Meatballs of other species did not amplify within a 40 cycle PCR reaction with the porcine-specific system, showing the high specificity of the method. Amplification of endogenous control revealed the presence of good quality DNA in all samples. While no significant difference was observed in the endogenous Ct values of the same species, some differences between the species were demonstrated.

Rojas et al. (2010) obtained variable results while quantifying game bird species in different commercial samples. They attributed these to a variation in factors, such as meat quality, tissue type, matrix composition and levels of processing. The present assay effectively minimized these issues by taking into account all the potential factors, such as tissue-composition, typical additives, and processing treatments of a typical commercial meatball. The real-time PCR assays developed by Rojas et al. (2010) for game bird determination were not optimized for a particular commercial meat product. They made a binary meat mixture of different percentages without considering the effects of typical ingredients found in a particular meat product. The present assay not only considered all the typical factors found in a meatball formulation during the process optimization but also limited the application of the developed assay to quantify pork only in meatball formulations. Thus, a high precision and accuracy of the assay were a logical outcome.

Fig. 3. RFLP analysis of PCR products before and after AluI-digestion. In gel image (A) shown are: L = DNA ladder, 1-12 = PCR products from meatballs of chevon (1, 2), mutton (3, 4), chicken (5, 6), beef (7, 8), beef with 10% pork (9, 10), and pork (11, 12) before and after AluI-digestion. The electropherograms of the respective PCR-products are shown by labels (B).
3.7. Product authentication by RFLP analysis

Although PCR has become an essential and daily performed experimental technique in bio-analytical, clinical and research laboratories, it is yet to be accepted as a definitive analytical method due to certain “difficult-to-control” features of the amplification process itself (Yang, Kim, Byun, & Park, 2005). These features mainly originate from the simultaneous amplification of small contaminants at the high magnitudes producing artifacts in the final results. However, the artificial PCR product can be easily distinguished from the original one by RFLP analysis if restriction sites are present in the real PCR products (Ali, Hashim, Mustafa, & Che Man, 2011). In-silico digestion of the 109-bp ampiclon of porcine cytb gene by NEB cutter version 2.0 reflected two-Alul sites inside the ampiclon with potential fragment length of 27, 33 and 49 bp. Endogenous control (141-bp) also carried one-Alul site within it with fragment length of 14-bp and 127-bp. Analysis of Alul-digest by bioanalyzer exhibited 27, 33, 49 and 126-bp fragments both in the gel-image (Fig. 3(A)) and electrophrograms (Fig. 3(B)), authenticating the originality of the PCR-products. The 14-bp fragment, probably, merged with the lower marker (15 bp) of the DNA ladder.

4. Conclusion

A real-time PCR assay suitable for the determination of adulterated pork in meatball formulations of 5 terrestrial meat species was developed. The method considered all the potential factors such as tissue-composition, additives, and processing treatments used in a typical commercial meatball while performing model experiment for process optimization. The assay showed 94–100% recovery of pork DNA in beef meatballs with 0.01–100% of spiked pork, showing a strong agreement with the actual values. The detection limit of the assay was 0.01% of pork in beef meatballs with a very low relative error (≤ 6%). High precision was confirmed by residual analysis and also by enough replicates in each measurement. Analysis of 45 commercial meatballs of various meat species revealed high specificity and reproducibility of the assay in quantifying pork in meatball formulations. Authenticity of the assay was further verified by RFLP analysis. The method has a strong potential to be used by regulatory and enforcement bodies and quality control laboratories for Halal and Kosher food verification and certification.

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