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Research Article

Quantitative Detection of Beef and Beef Meat Products Adulteration by the Addition of Duck Meat Using Micro Drop Digital Polymerase Chain Reaction

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A single-copy specific primer was designed based on beef and duck samples and through drop digital polymerase chain reaction (ddPCR) for the quantitative analysis. Results revealed that the primers had no specific amplification with sheep, chicken, pork, or other species. Both the relationships between meat weight and DNA weight and between DNA weight and DNA copy number (C) were nearly linear within the dynamic range. To calculate the original meat weight from the DNA copy number, the DNA weight was used as the intermediate value to establish the following formulae: Mbeef = 0.058C − 1.86; Mduck = 0.0268C − 7.78. To achieve a good quantitative analysis, all species used in the experiment were made of lean meat. The accuracy of the method was verified by artificial adulteration of different proportions. Testing of the commercial samples indicated that adulteration is present in the market. The established digital PCR method provided an effective tool for monitoring the adulterated meat products and reducing the adulteration in the market.

1. Introduction

At present, many businesses that aspire economic benefits sell cheap meat (e.g., pork, chicken, and duck) as high-priced meat (e.g., beef and mutton). Such business behavior not only deceives consumers but also affects the credibility of businesses. In addition, adulteration with inedible meat is harmful to human health. The identification of adulterated meat cannot be done by the naked eye and requires technical approaches.

These technical strategies include five types: chromatography, electrophoresis, spectroscopy, immunochemical techniques including enzyme-linked immunosorbent assay (ELISA) kits, and deoxyribonucleic acid- (DNA-) based techniques [1]. DNA testing is a commonly used method of testing meat. Microscopic observation and protein detection are hindered by their uncertainty, long processing time, and heavy workload. Polymerase chain reaction (PCR) technology is a good method that exhibits sensitivity and accuracy [2]. Although common PCR and fluorescence PCR are widely used in the qualitative detection of meat and meat products [2, 3], they cannot be used for quantitative detection. Vogelstein and Kinzler [4] proposed digital PCR in the late 20th century. Digital PCR performs an amplification reaction by dispersing the diluted sample into each individual reflecting unit. Each reaction unit includes one or more nucleic acid sequences, and then the fluorescent signal is read "yes" or "no" as the output. Calibration material was unnecessary, and the number of nucleic acids in the original sample was calculated by Poisson's distribution formula. Morisset et al. [5] used digital PCR (dPCR) and quantitative PCR (qPCR) to detect the MON810 gene in maize. The results showed that the sensitivity, repeatability, and tolerance to inhibitors of dPCR were higher than those of qPCR. Many studies have utilized dPCR to detect the gene expression

of complex samples, and the dPCR results have demonstrated advantages in this aspect [6–12]. Reid et al. [13] used dPCR to study the V600E and V600K mutations of the BRAF gene in circulating tumor cells of patients with malignant melanoma. The detection limit was 0.0005%, which greatly reduced the threshold of the lower limit of detection. The current application of dPCR in medical research to detect disease-related rare mutations in the serum, cells, and tissues of patients has achieved initial success, thus providing an important reference for medical diagnosis and treatment [14–17]. The measurement accuracy and sensitivity of dPCR are significant improvements of those of the two previous generations of PCR technology [18], making dPCR a true quantitative nucleic acid quantification technique [19]. Digital PCR is widely used in gene expression analysis [20], copy number variation analysis [21], genetically modified food testing [5], clinical diagnosis [22, 23], environmental microbiological tests [24], and so on. This strategy has promising prospects in a wide range of applications. In this study, a dPCR detection method was established by the linear curve between the quality of raw meat and its DNA content and then by establishing the linear curve between the DNA content and the number of DNA amplified copies. DNA concentration was used as the intermediate conversion value, and it was utilized to detect the contents of duck and beef meat to provide a scientific basis for the quantitative detection of meat products; also, it is a quick and simple method for testing department.

2. Materials and Methods

2.1. Experimental Materials. A total of 46 samples were purchased from major supermarkets and farmers markets in Shijiazhuang, China, and included fresh lean meat from beef, mutton, pork, duck, chicken, beef balls, bulk sauce beef, beef jerky, beef pellets, beef sticks, cowhide skins, spiced beef, prepackaged sauce beef, beef floss, prepackaged beef rolls, bulk beef rolls, children's steaks, bulk steaks, duck blood tofu, calf, duck wings, shredded duck, duck neck, and other meat products. Primers and probes were acquired from Shanghai Bioengineering Technology Co., Ltd. (China).

2.2. Laboratory Apparatus. Protease K was purchased from Tiangen Biochemical Technology Co., Ltd. (China). Sigma 1–15 pk refrigerated centrifuge was acquired from Sigma Company (Germany). Bio-Rad QX200 ddPCR Droplet Generator was obtained from Bio-Rad Company (USA). NanoDrop 2000 Micro Nucleic Acid Protein Analyzer was procured from American Thermo Company (USA). C1000 Touch Thermal Cycler Gene Amplification Instrument was bought from Bio-Rad Company (USA). The ME204/02 Electronic Balance was purchased from Mettler Toledo Co., Ltd. (Shanghai, China).

2.3. Experimental Method

2.3.1. Preparation of Meat Samples. Raw meat and commercially available meat products were obtained from the local supermarket. Fresh lean meat and commercial products were separately minced and dried in a baking oven (101-3AB; China) at 80°C for 72 h [25]. The dried samples were shredded again by a grinder and then minced to a superfine powder in liquid nitrogen by using a pestle and mortar [26]. Proportionally adulterated and commercially available samples were prepared separately to avoid contamination.

2.3.2. Beef and Duck Primer Design. Primers were designed based on the alignment of conservative region from beef and ducks. The 5′-end was modified with a FAM fluorophore, and the 3′-end was modified with a BHQ1 nonfluorescent quencher. The working concentration of the primers and the probes was 10 pmol. The beef- and duck-based primer designs were synthesized by Köppel et al. [2] and Cheng et al. [27], respectively (Table 1).

2.3.3. DNA Extraction. For all samples, genomic DNA was extracted from 10–100 mg of powder by using the phenol/chloroform method [25]. Exactly 1000 μL of histiocyte lysis buffer (Tiangen, China) and 150 μL of proteinase K (Tiangen, China) were added to each sample. The sample was vortexed and incubated in a 65°C water bath for 120 min, with occasional shaking. The samples were added with an equal volume of phenol/chloroform, mixed, and then centrifuged at 12,000 rpm for 10 min. The aqueous (upper) layer was transferred to a clean tube. Then, the sample was added with an equal volume of chloroform, mixed, and then centrifuged for 5 min at 12,000 rpm. The aqueous (upper) layer was transferred to a clean tube. The previous step was then repeated. The samples were added with two volumes of ice-ethanol (100%) and one-tenth volume of 3 M Na acetate, mixed, incubated at −20°C overnight, and then centrifuged at 12,000 rpm for 30 min. The supernatant was removed, and the DNA pellet was washed twice with 75% EtOH and then centrifuged at 12,000 rpm for 5 min. The supernatant was removed, and the pellet was air-dried for 30 min in the ultraclean platform, resuspended in 100 μL ddH2O, and then stored at −20°C. This centrifugation process was conducted at 4°C.

2.3.4. Digital PCR Reaction Program. For the droplet digital PCR (ddPCR) reaction system, 1.2 μL of each primer (final concentration: 900 nM), 0.4 μL of the probe (final concentration: 250 nM), and 10 μL of ddPCR™ Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA, USA) were mixed. Then, 4 μL (30-fold diluted from the original DNA extraction sample) of the template DNA and 3.2 μL of nuclease- and protease-free water were added. Approximately 20 μL of the system and 70 μL of oil were added to the droplet card, which was placed into the droplet generator to facilitate the droplet treatment. Micro droplets were generated and then transferred to a 96-well plate for normal PCR reaction. PCR reaction conditions: 95°C for 10 min; 40 cycles of 94°C for 30 s, 62°C for 1 min, and 98°C for 10 min.

Table 1: Primers and probes used in the experiment.

Primer/probe	Sequence/labeling ($5' \longrightarrow 3'$)	Modifying groups
Beef F	GTAGGTGCACAGTACGTTCTGAAG	
Beef R	GGCCAGACTGGGCACATG	5′-FAM, 3′-BHQ1
Beef probe	CGGCACACTCGGCTGTGTTCCTTGC	
Duck F	GGAGCACCTCTATCAGAGAAAGACA	
Duck R	GTGTGTAGAGCTCAAGATCAATCCC	5′-FAM, 3′-BHQ1
Duck probe	TGGGAACAAGCATGAATGTAAGTGGATGGT	

2.3.5. Primer and Probe Specificity. The DNA templates extracted from sheep, chicken, pig, walnut, and soybean were used as the negative control, those from beef and duck were used as the positive control, and sterile double-distilled water was used as the blank control for the specific detection of the required primers and probes.

2.3.6. Establishment of Sample Quality and Copy Number Formula

(1) Establishment of the Relationship Curve between Sample Quality and DNA Content. The meat samples used for genomic DNA extraction weighed 10–100 mg for beef and 10–60 mg. The DNA concentration of each sample was measured using a NanoDrop 2000 spectrophotometer, and each measurement was conducted in triplicate per quality.

(2) Establishment of the Relationship Curve between DNA Content and DNA Copy Number. Establishing the relationship between sample DNA content and DNA copy number required the serial dilution of the desired genomic DNA. In the experiment, the dilution gradient of beef was 20–200 ng/μL and that of duck meat was 20–180 ng/μL. The correlation curve was analyzed in this range, and each measurement was triplicated for each gradient.

2.3.7. Proportionally Adulterated Model Detection. To verify the accuracy of this experiment, the beef and duck adulteration rates were set as follows: 9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, and 1 : 9. The genomic DNA was extracted using the method described in Section 2.3.3 and then diluted by 30 times, resulting in 4 μL for detection. Three replicates were performed for each adulteration ratio.

2.3.8. Commercial Sample Detection. Testing of the commercially available samples was carried out using the droplet quantitative assay method established in this article (duck blood tofu A, duck blood tofu B, duck calf, duck wing root, shredded duck meat, duck neck, tender duck blood tofu, duck liver, duck wings, sweet spicy duck neck, beef balls, bulk sauce beef, beef grain, beef sticks, cowhide leather, prepackaged sauce beef A, prepackaged sauce beef B, prepackaged sauce beef C, beef loose, prepackaged beef rolls, bulk beef roll, children's steak A, children's steak B, children's steak C, bulk steak, spiced beef slices, beef ham, spicy beef, fat cow rolls, fat beef pieces, frozen cooked beef slices, beef roll, high-quality beef, sirloin cut, black pepper steak, salad steak, value-for-money steak, breakfast beef patty, fat beef pieces A, fat beef pieces B, Inner Mongolia fat cattle, fresh fat beef slices, sirloin steak, beef jerky A, beef jerky B, and featured lamb roll).

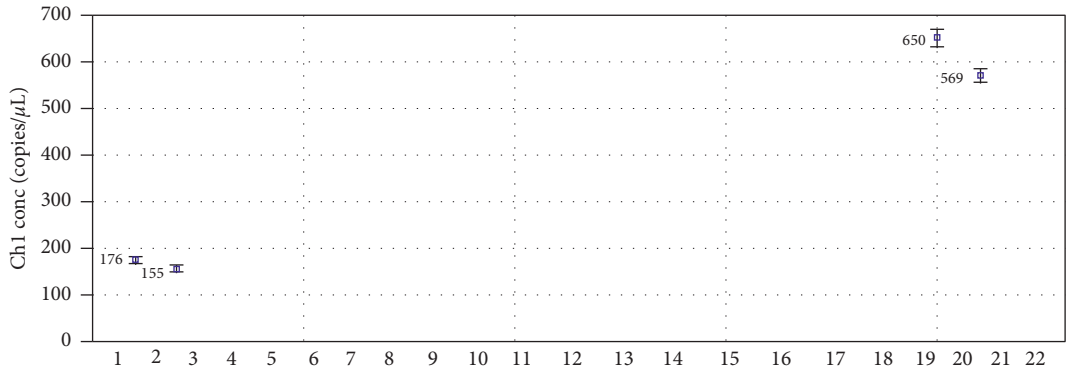
3. Results and Discussion

3.1. Specificity Detection. The genomic DNA templates extracted from sheep, chicken, pig, walnut, and soybean were used as the negative control, and beef and duck were used as the positive control. Water served as the blank control for the specificity detection. The results showed that the beef and duck primers had good specificity and were suitable for subsequent experiments (Figure 1).

3.2. Extraction and Determination of Genomic DNA from Beef and Duck Samples. The genomic DNA was extracted from pure meat samples of different qualities, and the results were determined by using NanoDrop 2000. This experiment measured optical density (OD) ratio, and the ratios were all in the range of 1.8 to 2.0, which was commonly accepted as good indicator for pure DNA [28]. Three replicates were performed in this step. The quality of the raw meat and the DNA content of the beef and duck samples displayed a good linear relationship within the gradient range of the weighed meat samples of different qualities (Figures 2 and 3).

3.3. Relationship between the DNA Content and Copy Number of the Beef and Duck Samples. After the relationship between the sample mass and its DNA content was determined, the genomic DNA was serially diluted [2] (beef dilution gradients: 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200; duck dilution gradients: 20, 40, 60, 80, 100, 120, 140, 160, and 180). The diluted genomic DNA was subjected to dPCR detection. Previous study [25] showed that more than 12000 effective microdrops can ensure the reliability of the experimental results. In our study, all samples' droplets exceeded 12,000, which satisfied the experimental requirements. The two species of beef and duck presented a good linear relationship in this range (Figures 4 and 5). The correlation coefficient of beef was $R^2 = 0.9992$ and that of duck was $R^2 = 0.9972$.

3.4. Establishment of Relationship between the Meat Quality and Copy Number of the Beef and Duck Samples. On the basis of the established relationship between the two species with DNA as the intermediate value, the relationships between meat quality and DNA copy number were derived as



1-2 beef primer template, 3-4 beef primer-sheep template, 5-6 beef primer-pork template,
7-8 beef primer-chicken template, 9-10 beef primer-duck template, 11-12 duck primer-beef template,
13-14 duck primer-sheep template, 15-16 duck primer-pork template, 17-18 duck primer-chicken template,
19-20 duck primer, 21 template, 22 negative

Figure 1: The specificity detections of the beef and duck primer-probe system.

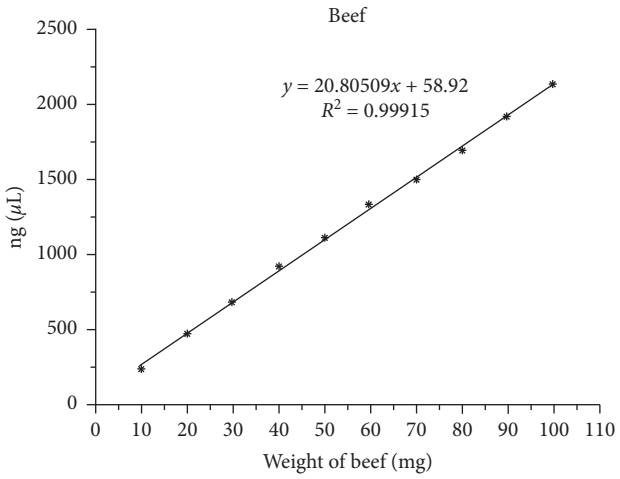


Figure 2: The relationship between the quality of raw meat and the DNA content of beef samples.

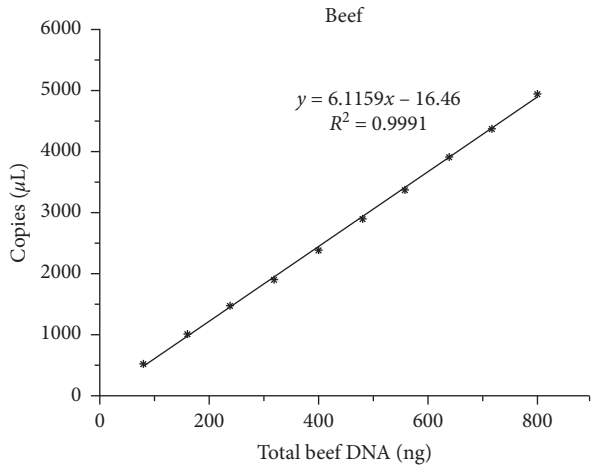


Figure 4: The relationship between the DNA content and the copy number of beef samples.

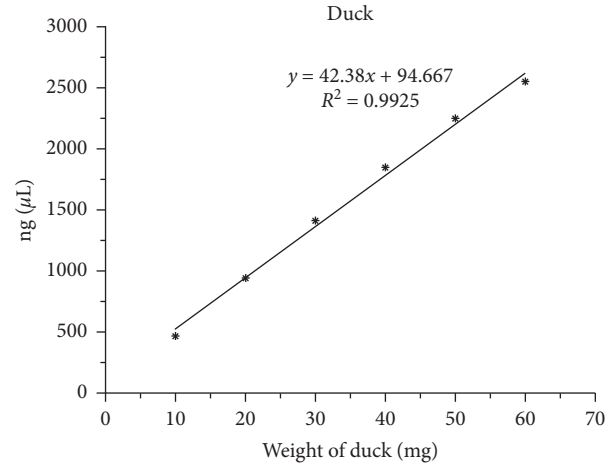


Figure 3: The relationship between the quality of raw meat and the DNA content of duck samples.

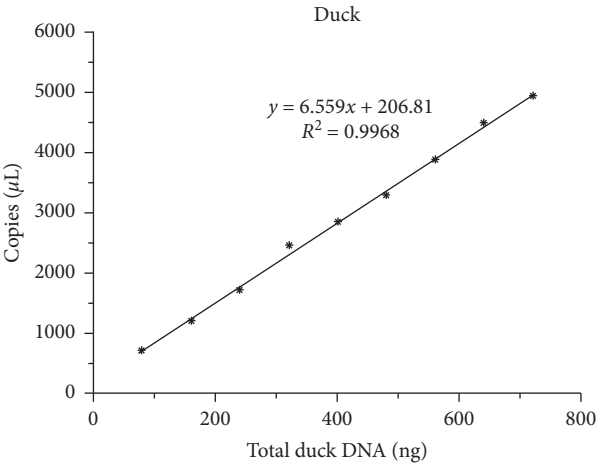


Figure 5: The relationship between the DNA content and the copy number of duck samples.

Table 2: Detection by digital PCR of mixed samples with known adulteration ratios.

Number	Beef mass (mg)	DNA copy number (copies/μL)			Average value (copies/μL)	Coefficient of variation (%)	Measured cassava mass (mg)	Relative error (%)
1	5.0	134	129	142	135 ± 6.55	4.86	5.97	19.4
2	10.0	225	220	230	225 ± 5.00	2.22	11.20	12
3	15.0	339	302	289	310 ± 25.94	6.62	16.12	7.46
4	20.0	366	354	369	363 ± 7.94	2.19	19.25	−3.75
5	25.0	485	492	493	490 ± 4.36	0.89	26.60	6.4
6	30.0	535	554	540	543 ± 9.85	1.81	29.69	−1.03
7	35.0	620	618	628	622 ± 5.29	0.85	34.22	−2.22
8	40.0	715	720	710	715 ± 5.00	0.70	39.61	−0.98
9	45.0	799	800	815	805 ± 8.96	1.11	44.83	−0.38

Note. Average values are provided as mean ± standard deviation.

Table 3: Detection by digital PCR of mixed samples with known adulteration ratios.

Number	Duck mass (mg)	DNA copy number (copies/μL)			Average value (copies/μL)	Coefficient of variation (%)	Measured cassava mass (mg)	Relative error (%)
1	5.0	481	495	491	489 ± 7.21	1.47	5.35	7
2	10.0	638	632	653	641 ± 10.82	1.69	9.40	−6
3	15.0	849	860	856	855 ± 5.57	0.65	15.16	1.06
4	20.0	1094	1114	1101	1103 ± 10.15	0.92	21.80	9
5	25.0	1290	1276	1280	1282 ± 7.21	0.56	26.60	6.4
6	30.0	1379	1390	1395	1388 ± 8.19	0.59	29.44	−1.86
7	35.0	1601	1623	1609	1611 ± 11.14	0.69	35.42	1.2
8	40.0	1889	1901	1892	1894 ± 6.24	0.33	42.98	7.45
9	45.0	1917	1888	1922	1909 ± 18.36	0.96	43.40	−3.55

Note. Average values are provided as mean ± standard deviation.

Mbeef = 0.058C − 1.86 and Mduck = 0.0268C − 7.78, where C is the copy number (copies/μL) and M is the raw meat weight (mg).

3.5. Proportional Artificial Adulteration Model of dPCR Detection. The DNA results were good, such that the genomic DNA was extracted from the proportionate adulterated meat samples. Three replicates are set in each adulteration ratio, and the DNA values extracted between the three replicates are not too different. The adulterated meat samples were diluted by 30-fold, resulting in 4 μL for dPCR. The digital PCR results obtained using the formula which is shown in Figure 2 were not significantly different from the actual ones and showed that the overall relative standard deviation was small for different proportions of beef (19.4%) and duck (9.0%) adulterated meat samples (Tables 2 and 3). The results are consistent with the previous research [25]. Although a number of the relative standard deviations for the meat samples of individual artificial adulteration models were slightly higher, the actual quality and experimental measurements were nearly the same, and the relative error rate of detection was below 20%.

3.6. Digital PCR Detection of Commercial Samples. The samples used in this experiment were collected from supermarkets and farmers markets. With the advancements of modern processing technologies, various types of commercially available meat products have increased, and the real species of processed products is not well identified. Identifying flesh varieties by appearance alone is difficult, thereby allowing unscrupulous producers to take advantage of unknowing consumers by using various spices and additives. Duck and beef products were tested by dPCR, and uneven results were obtained (Table 4).

The results showed that duck blood tofu A has the highest content of duck meat (42.29%), and more than half of commercially available duck samples contain less than 20% duck meat. In commercially available beef samples, the content of beef in beef balls, beef loose, and spiced beef slices were less than 0.5%, respectively. Even more surprising, the content of beef in beef balls is 0. Among the 46 samples tested, 28 samples' (60.87%) meat content of meat type marked in label was less than 20%, which indicated that meat adulteration is a widespread problem in the retail market. So, the development of reliable, precise, efficient, and rapid technique for routine quality control assessment of meat and meat products is indispensable. Prepackaged sauce beef was experimentally measured to contain the highest beef content of 9.45%. Prepackaged beef contained a small amount of beef, which was added with gel and other substances. The duck-derived primer had a copy number of zero, indicating that no substance was added to the duck. The bulk sauce beef with duck-derived primer was 4.09%, indicating that the duck ingredients were added. Most of the experimental results for beef rolls and steak products indicated the

Table 4: Adulteration detection of commercial samples.

Commercial sample	Beef (%), $n = 3$	Duck (%), $n = 3$
Duck blood tofu A	0	42.29 ± 2.61
Duck blood tofu B	0	4.28 ± 0.23
Duck calf	0	5.55 ± 0.17
Duck wing root	3.36 ± 0.18	19.75 ± 1.30
Shredded duck meat	0	13.38 ± 0.36
Duck neck	0	18.63 ± 0.93
Tender duck blood tofu	0	34.64 ± 1.01
Duck liver	0	0.072 ± 0.01
Duck wings	0	13.87 ± 0.53
Sweet spicy duck neck	0	4.33 ± 0.27
Beef balls	0	5.81 ± 0.27
Bulk sauce beef	4.23 ± 0.42	0
Beef grain	4.29 ± 0.21	0
Beef sticks	12.64 ± 0.70	0
Cowhide leather	73.42 ± 0.90	0
Prepackaged sauce beef A	4.58 ± 0.31	0
Prepackaged sauce beef B	4.11 ± 0.14	0
Prepackaged sauce beef C	9.45 ± 0.71	0
Beef loose	0.15 ± 0.01	0
Prepackaged beef rolls	57.30 ± 1.38	0
Bulk beef roll	70.93 ± 1.83	4.07 ± 0.23
Children's steak A	53.36 ± 1.29	0
Children's steak B	29.63 ± 1.21	0
Children's steak C	13.22 ± 1.04	0
Bulk steak	69.89 ± 1.74	0
Spiced beef slices	0.027 ± 0.005	0
Beef ham	5.04 ± 0.42	0
Spicy beef	3.01 ± 0.06	0
Fat cow rolls	3.65 ± 0.26	0
Fat beef pieces	4.29 ± 0.25	0
Frozen cooked beef slices	40.89 ± 1.23	0
Beef roll	11.13 ± 0.55	0
High-quality beef	14.55 ± 1.01	0
Sirloin cut	49.24 ± 1.11	0
Black pepper steak	28.53 ± 0.92	0
Salad steak	27.02 ± 1.61	0
Value-for-money steak	28.07 ± 0.97	0
Breakfast beef patty	6.38 ± 0.49	0
Fat beef pieces A	52.25 ± 2.31	0
Fat beef pieces B	40.54 ± 1.28	0
Inner Mongolia fat cattle	13.63 ± 1.10	0
Fresh fat beef slices	11.89 ± 0.34	0
Sirloin steak	54.52 ± 1.45	0
Beef jerky A	14.84 ± 0.29	0
Beef jerky B	57.47 ± 2.02	0
Featured lamb roll	42.51 ± 0.72	0

Note. The total mass of the commercial sample is 50 mg. Values are provided as mean ± standard deviation.

presence of beef. Some beef products are adulterated with duck ingredients. However, some products contain a large number of duck meat ingredients, whereas the real composition of beef is very small. Some businesses mislabel because of carelessness. Such a phenomenon was observed in this experiment, in that the product did not match the actual label. The sheep primer and bovine-derived primer test results were 0 and 42.51%, respectively. The duck-derived primer was 0. These findings indicated the incorporation of producing cheap meat, such as pork, chicken, and duck, presents no problem aside from careless mislabeling. The testing of the commercially available samples verified the feasibility of this method once again to provide a scientific basis for the inspection department.

4. Conclusions

After the horse meat crisis in Europe, adulteration issues of meat products have become incessant. Food types are complex and diverse, and different tissues vary considerably:

(1) In this study, the two selected species were tested based on the advantages of dPCR for trace analysis and the design of the primers on single copies of the species. The primers exhibited good specificity. This article established the relationship between raw meat quality and copy number curve. Quantitative analyses were performed on beef and duck meat. The two standard curves established for beef and duck meat were good, and R2 was above 0.99

(2) By using the established dPCR detection method, the genomic DNA was extracted from the meat samples added in different proportions. Then, quantitative experiments were performed on the extracted genomic DNA. The maximum mass ratio of beef is 19.6% and the maximum mass ratio of duck is 9%. Slight discrepancies were recorded between the theoretical and actual values of the meat composition in beef and duck meat. This method can be used to monitor the authenticity of beef slices in the market

(3) Digital PCR has a high sensitivity and short processing time. In this experiment, 46 commercial samples were tested by this method. There are 2 batches of obviously adulterated samples in the 46 commercial samples tested in this paper, which indicates that adulteration exists in the market. This method can be used for quantitative detection. The incorporation of cheap meat into expensive meat varieties is a means to attain tremendous economic benefits in the existing market. Although adulterated fake meat products do not bring harm, they still present consumer deception and credibility issues. The established method provided a scientific basis for reducing this adulteration phenomenon.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this work.

Authors' Contributions

Wei Zhou and Zhisheng Zhang carried out the concepts, design, definition of intellectual content, literature search, data acquisition, data analysis, and manuscript preparation.

Chen Chen, Jia Chen, and Yan Zhang provided assistance for data acquisition, data analysis, and statistical analysis. Chen Chen drafted the manuscript. Yongbo Li and Zan Wang carried out the literature search and data acquisition. Xian Li performed the manuscript review. Yongyan Li collected the samples. All authors have read and approved the content of the manuscript.

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