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QUANTITATIVE DETERMINATION OF PORK MEAT RESIDUE IN CATTLE MEAT MIXTURES USING DROPLET DIGITAL PCR

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ABSTRACT

Meat adulteration is an important economic and social problem worldwide. Therefore, the accurate identification and quantification methods for species substitutions in meat products are needed. In this study, a precise quantitative method was performed to identify the minimum content of pork (*Sus scrofa*) meat in the mixture of pork: cattle (*Sus scrofa :Bos taurus*) using the droplet digital Polymerase Chain Reaction (ddPCR) method. Experiments were conducted by using a series of dilutions for heat-treated and raw meat mixtures. The detection limits in DNA fragments of this study were 0.65 copies/µL for heat-treated and 0.1 copies/µL for raw meat samples. Based on these results, the sensitivity explaining minimum pork meat in meat mixtures was 0.1% for heat-treated and 0.001% for raw samples. The results showed that the ddPCR method is effective for identifying and quantifying pork meat in meat products and has potential to be applied for other meat species also.

1. Introduction

Determining the origin and contents of meat and meat products is an important issue worldwide because of authenticity problems. The most frequent meat adulteration is the use of different animal origins as a meat source. The use of different meat origins without declaring them on the label causes some religious and health problems and creates unfair competition. Consequently, species identification is gradually gaining importance (Hossain et al. 2019).

The need for safe and rapid methods for determining animal species in foods is becoming crucial. The methods used for meat species' identification are generally protein and DNA-based analyses. The most frequent protein-based methods used for species identification in meats products are electrophoretic, immunochemical and chromatographic (Alikord et al. 2018). However, these methods have some disadvantages because of the denaturation of proteins during the heating process (Fajardo et al. 2010). The DNA-based methods are more advantageous from this point because the DNA present in animal tissues could resist to some physical factors such as heating process. DNA molecules are more resistant to the heat process in comparison to proteins and large numbers of DNA cells led to increase the efficiency of DNA based methods (Alikord et al. 2018). Moreover, the amount of mitochondrial DNA (mtDNA) changes variably according to the tissue type used for a species' identification application. Therefore, the use of nuclear DNA is reported as more effective than mtDNA for reliable

quantification results (Floren et al. 2015). Spectroscopic techniques are used also for the determination of authentication in meat products being rapid and non-destructive methods (Sajali et al. 2021). However, they have some disadvantages of showing lack of specificity for identification of resembling meat species and heat-treated materials due to the denaturation of proteins (Fajardo et al. 2010).

DNA-based polymerase chain reaction (PCR) technique is a rapid, sensitive, highly specific, and cheaper alternative method used to identify animal origins even in processed foods (Amaral et al. 2015; Soares et al. 2013). However, quantitative data about the target DNA is another important point for evaluating the adulteration. Improvements in PCR techniques have created the real-time PCR (qPCR) which can detect quantitatively the presence of DNA (Sajali et al. 2021). The real-time PCR technique allows monitoring and measuring the amplified products along each amplification cycle. The presence of PCR products is identified with fluorescent molecules during the real-time process (López-Andreo et al. 2005). For this purpose, two different fluorescent chemistries have been used, namely universal fluorescent dyes, such as SYBR green, and sequence-specific DNA probes like the hydrolysis TaqMan dual labelled probes. Recently, the real-time PCR method was used successfully for the species identification of some different meat species (Kang et al. 2021; Köppel et al. 2020; Li et al. 2021; Liu et al. 2021; Ren et al. 2017).

Droplet digital PCR (ddPCR) has emerged in recent years as an advanced technique that enables detecting and quantifying DNA at trace levels. The principle of this method is to count the number of identifications in a sample by using excessive dilutions of nucleic acids and Poisson statistics into multiple individual PCR amplification cells. In every cell, individual PCR assays are performed, and positive PCR amplifications are monitored with fluorescent target-specific probes. It is reported that the ddPCR method is more sensitive and precise than other quantitative real-time PCR methods (Basanisi et al. 2020; Cao et al. 2020; Dimond et al. 2022). ddPCR permits quantifying the absolute number of nucleic acids in several thousand of individual compartments by portioning and without the use of standard curves. In addition, ddPCR permits the possibility of determining and quantifying very few nucleic acid contents in a sample (Hudecova 2015). The ddPCR has been used in some studies for the development of methods for determining meat authenticity. Ren et al. (2017) compared the two methods (real-time PCR and ddPCR) for determining meat adulteration and they reported that the ddPCR method is more accurate and easier compared to real-time PCR. In their study, they detected 1% (w/w) sheep and 0.1% chicken meat in meat mixtures. Wang et al. (2018) developed a ddPCR method for determining goat meat and sheep meat derivatives in commercial meat products. Floren et al. (2015) reported a limit of quantification (LOQ) of 0.01% and a limit of detection (LOD) of 0.001% for beef, horse meat and pork in processed meat products. Cai et al. (2017) used a duplex ddPCR method to detect and quantify beef and pork materials in meat products. They reported LOD/LOQ of 0.1 ng/μL for beef and 0.5 ng/μL for pork. Noh et al. (2019) applied ddPCR for determining Alaska pollock (*Gadus chalcogrammus*) content in seafood products.

The real-time PCR technique has some limitations related to the accurate measurements. The impurities and contaminant levels in DNA solution can affect the amplification efficiency and the copy numbers based on Ct values from real-time PCR (Hudecova 2015). Digital droplet PCR (ddPCR) exhibits an effective single molecule counting principle to detect very small amounts of genetic material, thus providing precise and sensitive determinations that can eliminate the effect of contaminants without the use of standard curves. Besides, the reports on the application of ddPCR to the quantification of meat species in food is still limited. Hence, the objective of this study was to determine the minimum quantity of pork meat in heat-treated and raw pork:cattle meat mixtures of different concentrations using

ddPCR as an alternative new method for quantitative identification of meat species.

2. Materials and methods

2.1. Materials

Fresh lean cattle (*Bos taurus*) meat was provided by a local butcher (Yamaner, Bolu, Turkey) and pork (*Sus scrofa*) meat was provided by a local market (İstanbul, Turkey). They were immediately stored at -18°C in 1 kg plastic containers until use. All chemicals and reagents used were analytical grades.

2.2. Sample preparation

Frozen meats were thawed at 4°C and the fat and connective tissues removed. They were ground to 1-2 mm with a chopper. To avoid contaminations, the knives and the chopper were cleaned carefully and treated with DNA decontamination solution (20% bleach) after each grounding process between the meat species. Next, a series of pork:cattle binary meat mixtures were prepared at different ratios such as 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2.5%, 5% and 10% (Table 1). To provide the homogeneity, meat mixtures were homogenized separately in a laboratory blender (Waring Commercial 8010ES, USA) for 2-3 minutes. The knives and the container of blender were cleaned and treated with DNA decontamination solution (20% bleach) to remove residual DNA between samples. A total of 400 g of meat mixture was prepared for each group and divided into two equal parts. One part of the meat mixture was shaped into standard patties 4 cm in diameter and 0.5 cm in thickness for the cooking process. They were baked in an oven (Memmert UN-800) at 120°C (75°C internal temperature). The second part was used for the direct DNA isolation.

Table 1. Meat mixtures

No	% Cattle Meat	Cattle Meat (g)	Pork Meat (%)	Pork Meat (g)
1	0	0	100	200
2	90	180	10	20
3	95	190	5	10
4	97.5	195	2.5	5
5	99	198	1	2
6	99.5	199	0.5	1
7	99.9	199.8	0.1	0.2
8	99.99	199.98	0.01	0.02
9	99.999	199.998	0.001	0.002
10	99.9999	199.9998	0.0001	0.0002
11	99.99999	199.99998	0.00001	0.00002
12	100	200	0	0

2.3. DNA extraction

Before the DNA isolation, a 25 mg of meat sample from each meat mixture was homogenized with 75 µL PBS (Phosphate Buffered Saline) (pH: 7.3) in a 1.5 mL microcentrifuge tube. After the homogenization, the DNA isolation was performed according to the manufacturer's instructions (Qiagen DNeasy Blood & Tissue Kit, USA). The amount and quality of DNA were determined by measurement of absorbance at 260 nm and 280 nm using a nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). The ratio of absorbance of the A260/280 values which are between 1.7 and 2.0 was selected. The DNA samples of each group were diluted to 25 ng/µL in the elution Buffer AE (Qiagen DNeasy Blood & Tissue Kit, USA) and then were tested directly.

2.4. Primers and probes

Primers and probes were retrieved from Floren et al. (2015) (Table 2) to target the

chromosomal coagulation factor II (*F2)* gene to amplify fragments of 96 bp for and 97 bp for cattle and pork, respectively. All primers and probes were synthesized by Metabion GmbH (Germany). The hydrolysis probes were labeled with Carboxyfluorescein (FAM) as the indicator for pork and labeled with Hexachlore-6-carboxyfluorescein (HEX) for cattle. For verifying the specificity of the primers, BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) was used.

Table 2. Primers and probes sequencing used for ddPCR

Primer name[a,b]	Sequence (5´-3´)	Chromosome	Position Genomic[c]
Sus_F2_For	5'-CTGCCAGCGGGCTGGGAATA -3'	2	SSC2:17167390-17167410
Sus_F2_Rev	5'-GGAGTTGACTCTGGAATAAGAAAT TG -3'	2	SSC2:17167460-17167486
Sus_F2_FAM	5'-FAM-CGCCCCCGCCCCCAGGGTCT - BHQ1-3'	2	SSC2:17167438-17167457
Bov_F2_For	5'-CCTGTCTGCTGAGACGCCG-3'	15	BTA15:76998246-76998265
Bov_F2_Rev	5'- GTGGTAGAGTTGATTCTG GAATAGAAAGCAT -3'	15	BTA15:76998310-76998341
Bov_F2_HEX	5'-HEX -CCCCGCCACCCGCAGTGTCT-BHQ1-3'	15	BTA15:76998274-76998293

[a]F2: coagulation factor II (F2) gene
[b] Sus: pork; Bov: cattle
[c] Bovine genome: Btau_4.6.1; Porcine genome: Sscrofa_10.2

2.5. Droplet digital PCR and analysis

Following the DNA extraction and concentration adjustment for the PCR assay, the digest step, droplet generation, PCR step, and droplet reading were performed, respectively. Samples of meat mixtures were prepared in a manner so that the DNA concentrations of each sample were 25 ng/µL. Firstly, 0.5 µL *Bam*HI (New England Biolabs GmbH, Germany) containing 0.055 µL BSA (Bovine Serum Albumin) (Thermo Scientific, 23209, USA) in 11 µL 1x ddPCR supermix (#1863024, BioRad Laboratories, USA) was added to the 1 µL template DNA and incubated at 37°C for 1 hour. After the digestion, 0.9µM of each primer and 0.25µM of each hydrolysis probes were added. A total of 10 µL of the dUTPs enzyme solution was added and the final volume was completed with 22µL of nuclease and protease-free water (AppliChem GmbH, Germany).

A total of 20 µL from each mixture of 12 different groups and non-template control (NTC) samples were loaded using a multichannel pipette into an eight-channel single-use droplet generating generator cartridge (#1864007, BioRad Laboratories, USA). Droplets were generated from a droplet generator (QX200; BioRad Laboratories, USA). The droplets are aqueous substances surrounded by oil and contain a surfactant that prevents oil droplets from clustering together. After droplet generation, the droplets were transferred gently onto a 96-well plate (40 µL for each well) with a multichannel pipette and the PCR amplifications were performed using a thermal cycling protocol in a thermal cycler (C1000 Touch, BioRad, USA) according to the amplification protocol of Floren et al. (2015) stated in Table 3. After the PCR amplification, the plate containing the droplets was placed in the droplet reader (QX200, BioRad Laboratories, USA), which examined each droplet by counting positive reactions one-by-one using the two-color determination method (FAM and HEX) for determining the number of species at the lowest concentration in the

mixture. Positive droplets which comprised at least one replica of the target DNA molecule, represented an increased fluorescence compared to negative droplets. The measurement of the positive and negative droplets for each sample was performed using the Absolute Quantification (ABS) method Quanta Soft™ 1.7.4. software which adapts the results to the Poisson algorithm for defining the preliminary concentration of the target DNA molecules by copies/µL (Biorad 2018).

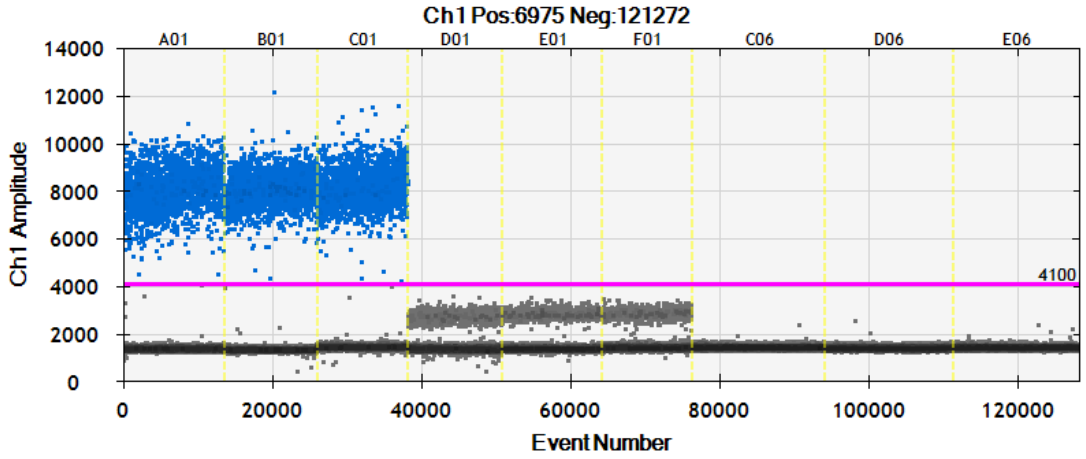
Table 3. Reaction conditions of ddPCR

Step	***F2***
Initial denaturation (°C/min)	95/10
Cycles	50
Denaturation (°C/s)	95/30
Primer annealing (°C/s)	55/10
Primer extension (°C/s)	68/20
Inactivation (°C/min)	98/10

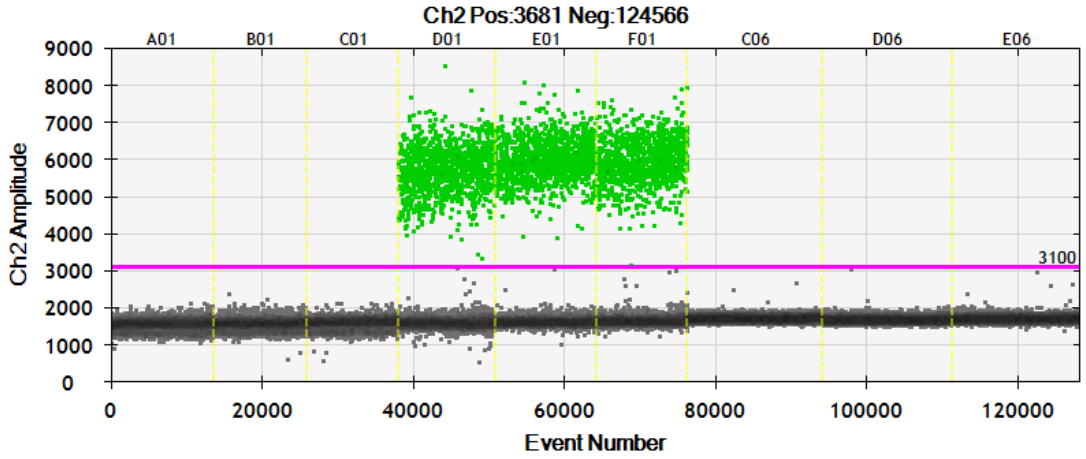
3. Results and discussions

3.1. The tests of specificity of primers and probes

The specificity assays for primers and probes have been performed by Floren et al. (2015) by using DNA from different horse, cattle, and pig breeds. In addition to this, we made some ddPCR analysis for investigating the probable cross-contaminations. As shown in Fig. 1, there is no cross-contamination between the amplitude of droplets (Lanes A01, B01, C01) showing the reaction signals of 100% pork DNA (FAM, blue-stained). In Fig. 1a, there is no cross-contamination also between the amplitude of droplets (Lanes D01, E01, F01) showing the reaction signals of 100% cattle DNA (HEX, green-stained). On the other hand, there were not any FAM or HEX fluorescence signals in (Lanes C06, D06, and E06) showing the reaction of signals in the NTC samples (Fig. 1b). The results showed that this analysis can be performed for reliable quantification of cattle and pork species in meat mixtures for sensitivity of primers and probes.



(a)



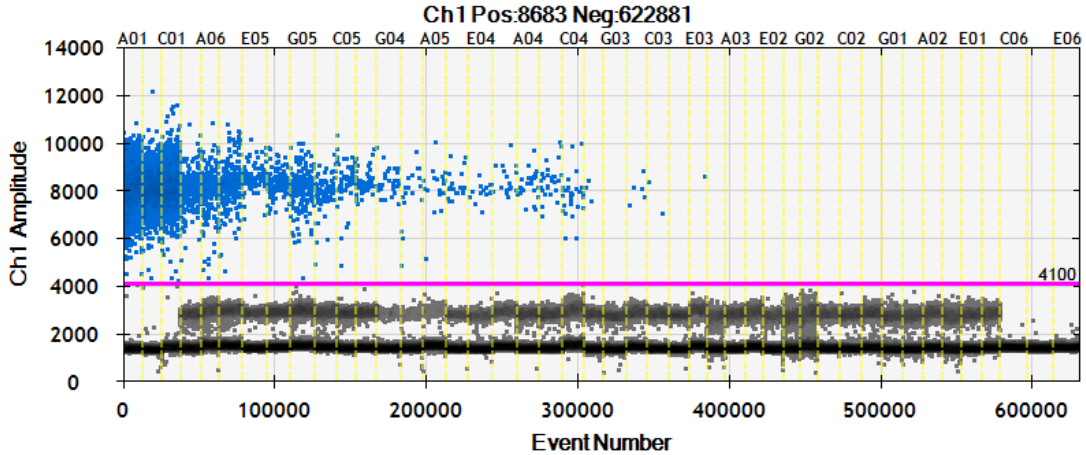
(b)

Figure 1. The specificity results of ddPCR assays. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: A01, B01, C01: 100% pork; D01, E01, F01: 100% cattle; C06, D06, E06: NTC.

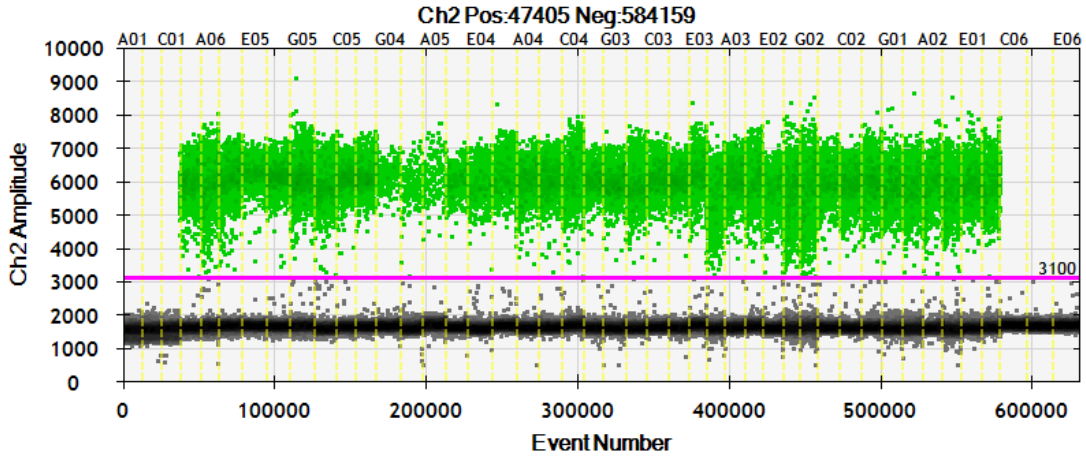
3.2. ddPCR results for raw meat mixtures

At this study, the copy numbers were obtained by ddPCR assays based on the weight proportions of meat mixtures. The analysis was performed in triplicate for each sample. At the end of the ddPCR process for the raw meat mixtures, the numbers for the mean of the droplets were 14,075 and standard deviations were 1785 droplets, respectively (Fig. 2). As a result of the ddPCR process, 0.1 copies/µL of the sample 0.001% pork meat was observed as a minimum copy number corresponding to 2 positive droplets (Fig. 2a).

According to the ddPCR analysis results of droplets for the raw meat mixture sample of 0.001% pork, 2 positive droplets were detected in 14,213 droplets as shown at the lane C03 (Fig. 2a). Also, in the parallel analysis 1 positive droplets was detected in 11,579 droplets as shown at the lane E03 (Fig. 2a).



(a)



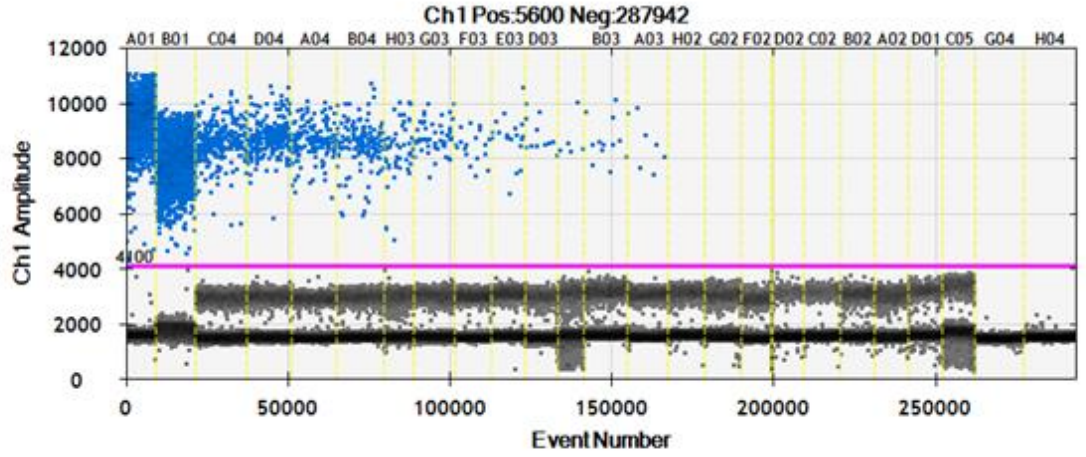
(b)

Figure 2. The results of ddPCR assays in raw meat samples. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: C03: 2 positive droplets detected; E03: 1 positive droplet detected; C06, E06: NTC.

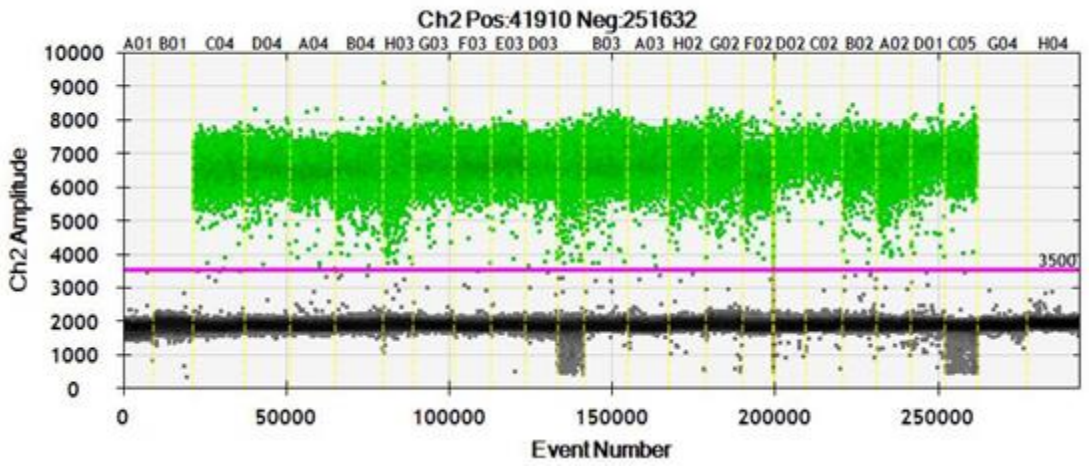
The means and standard deviations of ddPCR results for raw meat mixtures were given in Table 4. The experiments conducted for NTC samples were resulted in no positive droplet (no signal) that showing no contamination in the experiment (Fig. 2a and Fig. 2b, Lanes C06 and E06; Table 4).

3.3. ddPCR results for heat-treated meat mixtures

The copy numbers were obtained by ddPCR assays based on the weight proportions of heat-treated meat mixtures. The analysis was performed with a duplicate for each sample. At the end of the ddPCR process for heat-treated meat mixtures, the numbers of the mean of the droplets were 13,852 and standard deviations were 1375 droplets (Fig. 3). As a result of the ddPCR process, 0.65 copies/µL of the sample 0.1% pork meat was observed as a minimum copy number corresponding to 7 positive droplets (Fig. 3b).



(a)



(b)

Figure 3. The results of ddPCR assays in heat-treated samples. (a) The ddPCR assays for pork; and (b) The ddPCR assays for cattle. The horizontal axis represents the event number of the meat mixture. The vertical axis represents the amplitude of samples. Lanes: A03: 7 positive droplets detected; B03: 12 positive droplets detected; G04, H04: NTC.

According to the ddPCR analysis results of droplets for the heat-treated meat mixture sample of 0.1% pork, 7 positive droplets were detected in 12,666 droplets as shown in the lane A03 (Fig. 3a). Also, in the parallel analysis 12 positive droplets were detected in 13,632 droplets as shown in the lane B03 (Fig. 3a). This result could be explained by the denaturation effect of heat treatment by the cooking process on DNA fragments. The means and standard deviations of ddPCR results for heat-treated meat mixtures were given in Table 5. The experiments conducted for NTC samples were resulted in no positive droplet (no signal) that showing no contamination in the experiment (Fig. 3a and Fig. 3b, Lanes G04 and H04; Table 5).

Many research studies based on the qPCR or real-time PCR have been reported for species identification and quantification in meat products as follows. Ren et al. (2017) reported the detection of 1% (w/w) sheep meat in sheep: chicken meat mixture and 0.1% chicken in the meat mixture. They compared real-time PCR and ddPCR methods and they reported that the ddPCR method is more accurate and easier compared to the real-time PCR method. Ulca et al. (2013) reported a 0.1% detection limit of pork meat in raw and cooked beef, chicken and turkey meat products using commercial PCR kits. Ali et al. (2015) practiced a multiplex PCR assay to identify five meat species (cat, dog, pig, monkey, and rat) in meatballs (cooked at 121°C) and they reported a detection limit of 1%. Kesmen et al. (2009) performed a species-specific PCR analysis for the identification of pork, horse meat, and donkey meat in cooked sausages. The lower detection level that reported was 0.1% for each other. Köppel, Zimmerli, and Breitenmoser (2009) developed a quantitative multiplex PCR method for detection of pork, beef, chicken, turkey, horse meat, sheep and goat meat and the minimum detection level reported by this study was 2%. Mousavi et al. (2015) reported a detection limit of 0.1% for identifying of chicken and donkey in raw meat samples due to a species-specific PCR study.

In our study, minimum detection of pork in pork:cattle meat mixtures were stated as 0.1% for heat-treated and 0.001% for raw samples. Compared to the results obtained by previous reported works based on the qPCR or real-time PCR, the results of our ddPCR study were more sensitive, especially for heat-treated samples.

Table 4. ddPCR analysis results of raw binary meat mixtures

Sample No	Pork meat (%)	Copies / µL		Copies / Well (20 µL)		Positives Droplets		Accepted droplets	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0*	-	-	-	-	-	-	17273.00	489.79
2	0.0000001	-	-	-	-	-	-	13307.00	678.05
3	0.000001	-	-	-	-	-	-	14245.33	889.02
4	0.00001	-	-	-	-	-	-	12033.00	1394.88
5	0.0001	-	-	-	-	-	-	12281.00	1415.62
6	0.001	0.09	0.09	1.80	1.71	1.00	1.00	13139.00	1382.62
7	0.01	0.23	0.21	4.60	4.26	2.67	2.52	13823.67	938.62
8	0.1	2.10	0.00	42.00	0.00	26.33	1.53	14823.67	1025.50
9	0.5	1.20	0.26	24.00	5.29	15.67	2.08	15565.67	1347.99
10	1.0	1.70	0.52	34.00	10.39	22.00	7.81	15273.67	743.98
11	2.5	5.36	0.67	107.33	13.32	62.67	13.43	13751.67	1528.57
12	5.0	11.70	4.07	234.00	81.46	156.33	52.60	15863.00	317.51
13	10	24.36	2.60	487.33	51.94	282.67	64.61	13665.67	1834.16
14	100	237.33	11.02	4746.67	220.30	2325.00	140.62	12741.00	848.46
	NTC	-	-	-	-	-	-	12735.00	670.35
	Total	18.94	1.30	378.78	25.91	192.96	19.08	14034.76	192.96

* : Cattle (100%)
SD : Standard deviation.
NTC: None Template Control.
- : not detection

Table 5. ddPCR analysis results of heat-treated binary meat mixtures

Sample No	Pork meat (%)	Copies / µL		Copies / Well (20 µL)		Positives Droplets		Accepted droplets	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0*	-	-	-	-	-	-	10917.50	277.89
2	0.0000001	-	-	-	-	-	-	11231.00	3003.79
3	0.000001	-	-	-	-	-	-	11836.00	927.72
4	0.00001	-	-	-	-	-	-	10484.50	235.47
5	0.0001	-	-	-	-	-	-	10408.50	958.13
6	0.001	-	-	-	-	-	-	11447.50	2704.68
7	0.01	-	-	-	-	-	-	11166.50	185.97
8	0.1	0.83	0.25	16.50	4.95	9.50	3.54	13149.00	683.07
9	0.5	0.90	0.14	18.00	2.83	7.00	2.83	8878.50	1747.26
10	1.0	2.20	0.42	44.00	8.49	20.50	3.54	11074.50	521.14
11	2.5	5.60	0.71	112.00	14.14	50.50	3.54	10808.50	2202.64
12	5.0	12.35	1.06	247.00	21.21	149.00	7.07	14323.50	516.90
13	10	20.30	1.41	406.00	28.28	254.50	6.36	14926.50	1413.51
14	100	284.00	5.66	5680.00	113.14	2309.00	325.27	10782.50	1707.66
	NTC	-	-	-	-	-	-	15577.50	495.68
	Total	21.75	0.64	434.90	12.87	186.67	25.15	11880.80	1172.10

* : Cattle (100%)
SD : Standard deviation.
NTC: None Template Control.
- : not detection

4. Conclusions

Regarding the authenticity problems in the world, the (qPCR) is already a more prevalent method for identification and quantification of meat species compared to ddPCR, because of its lower costs. Additionally, the ddPCR method would require more time which could be attributed to the droplet reader process for analyzing individual droplets. However, ddPCR provides the measurements with higher precision, sensitivity, and repeatability which is related to the number of partitions ensuring concurrent template amplification and of being capable of detecting the smallest traces of nucleic acids. This study was performed in order to identify minimum pork meat quantity in raw and heat-treated meat mixtures (cattle:pork) using an alternative ddPCR method. The main purpose of this study was to prove the usability of ddPCR for the quantification of meat species with higher precision and sensitivity. Consequently, the absolute limits of detection (aLOD) of the ddPCR were determined as 0.65 copies/µL and the absolute limits of quantification (aLOQ) of ddPCR were determined as 13 copies/µL for heat-treated samples. The (aLOD) results were defined as 0.1 copies/µL and the (aLOQ) results were defined as 2 copies/µL for raw meat samples. According to these results, the minimum pork meat concentration in meat mixtures (cattle:pork) samples was identified with a precision of 0.001% (for raw samples) and 0.1% (for heat-treated samples). Our study showed that ddPCR could be an effective alternative tool usable for law enforcement authorities to control and prevent food adulteration. Furthermore, this method has the potential of being adapted for the quantifying of various other meat species.

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