PRELIMINARY ESTABLISHMENT OF A MULTIPLEX PCR METHOD FOR THE IDENTIFICATION OF PORK AND BEEF MEAT BASED ON CYTOCHROME-B GENE

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ABSTRACT

Molecular species detection in food has become common in the last years. In this study, multiplex PCR (m-PCR) technique was applied to discriminate between the pork and beef with the aim of detecting the pork in food products made from beef. We developed a m-PCR protocol detected the presence of pork in food products made from beef based on *Cytochrome-b* gene by a general primer pair F and primer pair (R_P, R_C), specific two private with pig and cow. The m-PCR method was successfully designed with technical parameters, such as the annealing temperature of 59^0C and the final concentration of each primer in a reaction of 0,4µM. The minimum DNA concentration of pig and cow could be detected by m-PCR, which was 0,1ng/µl. This process was tested on 24 different beef sausage samples tagged no pork, resulting in 11/24 (46%) of samples, were found the presence of pork and 54% (13/24) of samples no beef and pork. According to this sequencing result that are completely accordant, we affirm primer-specific amplification in this study can be applied to experiment on large number of sample and the ton other types food made from beef.

Keywords: Beef; *Cytochrome-b*; discriminate; multiplex PCR; pork.

1. Introduction

Nowadays, as the demand for food and foodstuffs is increased, it is essential to ensure quality, hygiene and safety of food, in which meat-based products have been one of the top social concerns (Sawyer J et al., 2003). Meat products often come from one or several different types of meat. It is the variety in terms of appearance, quality and cost of processed meat products that caused the problem of trade fraud. The situation of fraud in the production and consumption of fresh meat, processed meat and consecutively-raging dangerous epidemics made the requirement of ensuring the honesty and safety of these products (Sónia Soares et al., 2013). This requires quality managers, market controllers and researchers to always find the methods to detect, distinguish quickly and accurately types of meat in products made from meat and poultry.

Polymerase chain reaction (PCR) is a common method used by many researchs in the world to amplify target DNA sequences of animal-derived ingredients (Lao Duc Thuan et al., 2014). Some target sequences are used as mitochondrial 16S rDNA (Lao Duc Thuan et al., 2014), mitochondrial 12S rDNA (Arun Kumar et al., 2012; Fajardo et al., 2010), *Cytochrome-b* gene of the mitochondrial genome (Abdul-Hanssan et al., 2014; Deepak Kumar et al., 2012; Md.Eaqub Ali et al., 2015; T.matsunaga et al., 1999). In this study, the Cytochrome b gene was selected as the target DNA sequence for m-PCR, *Cytochrome-b* gene is located on the mitochondrial genome which is used very often in studies concerning meat species identification and therefore sequence data of many vertebrates and nonvertebrate species are available. Mitochondrial DNA owns several advantages over nuclear DNA. Mitochondrial DNA is

presented in thousands of copies per cell and possesses many points of mutations allowing the discrimination of even closely – related species. Mitochondrial DNA is maternal inheritance and therefore is free of heterozygosity (Unseld M et al., 1995; Lockley A.K et al., 2000; Anita Spychaj 1 et al., 2009).

2. Materials and Methods

2.1. Material

Sample sources for DNA extraction: Positive controls included animal samples, beef and pork. Negative controls were shrimp, squid, fish, and chicken samples. The actual samples were random samples of beef sausage from different manufacturers purchased at supermarkets in Ho Chi Minh City.

Primers in m-PCR amplification: The amplifying primer pairs used to distinguish pork and beef were based on reference of previous studies, as detailed in Table 1. One common primer (F) and two reverse primers (R_P and R_C, specific for pork and beef respectively) were used (Abdul-hanssan et al., 2014; Deepak Kumar, S.P. et al., 2012). All primers used in the study were synthesized by Integrated DNA Technologies (IDT) - USA.

2.2. Methods

DNA extraction

The DNA extraction from all samples was performed according to the manufacturer's instruction provided using the Isolate II Genomic DNA Kit (Bioline, UK). **Step 1**. Sample preparation: place up to 25mg tissue into 1.5ml tube. **Step 2**. Sample pre-lysis: add 180µl Buffer GL + 25µl proteinase K solution, vortex, incubate 56^{o}C, 1hour, vortex. **Step 3**. Sample lysis: add 200µl Lysis Buffer G3 and vortex, incubate 70^{o}C, 10 min. **Step 4**. Adjust DNA binding conditions: add 210µl ethanol, vortex. **Step 5**. Bind DNA: load lysate, centrifuge for 1 min at 11.000. The DNA bound to the column has been washed in two centrifugation steps using two different washing buffers to improve the purity of the eluted DNA. **Step 6**. Wash silica membrane: 1st wash 500µl Wash Buffer GW1, centrifuge for 1 min at 11.000, then, 2nd wash 600µl Wash Buffer GW2, centrifuge for 1 min at 11.000. Dry silica membrane by centrifuging for 1 min at 11.000. **Step 7**. Elute DNA: add 100µl Elution Buffer G (70^{o}C), centrifuge for 1 min at 11.000. Isolated DNA.

m-PCR reaction

The 25µl reaction mixture was prepared in an Eppendorf tube containing 12.5µl of MyTaq HS Mix (Bioline, UK) (of all four dNTP and Taq DNA polymerase), 1µl of 0.4µM of each primer, 2µl of DNA template and ultrapure water up to 25µl. The thermocycler was programmed for 35-cycle m-PCR. m-PCR was optimized with different annealing temperatures. The optimal annealing temperature was 59°C for all primers, the following thermal cycle conditions were carried out: melting at 95^{o}C for 15s, annealing at 59^{o}C for 15s, extension at 72^{o}C for 10s. A final elongation phase was applied at 72^{o}C for 5min. Electrophoresis was run on agarose gel (1.5%) at 100 V for 40 minutes on a 12µl portion of the amplified DNA fragments. The resulting gel was stained with GelRed (TBR, Vietnam), visualized using a Gel.doc machine and sequencing at Nam Khoa Company, Vietnam.

Table 1

Cytochrome-b gene primers sequences (Sónia Soares et al., 2013; Haining He et al., 2015)

Name	Primer	Sequences (5' – 3')	Amplicon size (bp	Position
Common	F	ATCCGACACAACAACAGCATTCTCCT		
Pig	R_P	GCTGATAGTAGATTTGTGATGACCGTA	288bp	168-455
Cow	R_C	CTAGAAAAGTGTAAGACCCGTAATATAAG	164bp	168-331

3. Results and discussion

3.1. Results of theoretically testing characteristics of the primer pairs

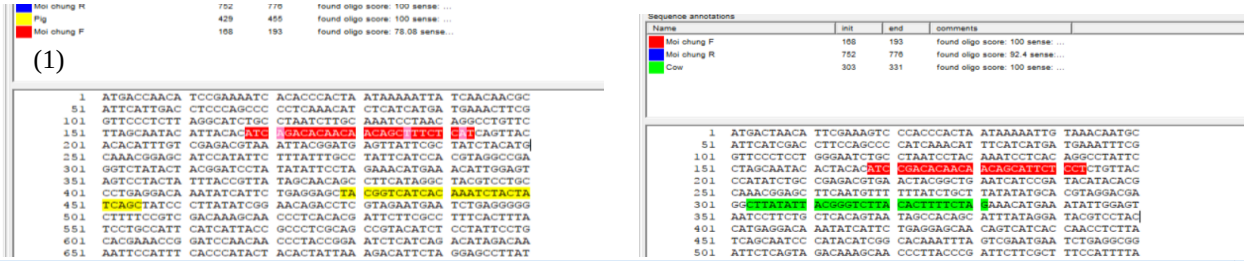
The physical characteristics of primers were evaluated by the IDT software described in Table 2.

Table 2

The physical characteristics of primers

Primer	Length (bp)	% GC	Tm (^{0}C)	1	2	3
F	26	46,2	60,4	1,28	-3,61	
R_P	27	40,7	56,2	0,54	-3,61	-7,19
R_C	29	34,5	53,2	0,58	-4,16	-5,12

Note: Tm: melting temperature; (1) ΔG of hairpin-loop (kcal.mole-1); (2) ΔG of self-dimer (kcal.mole-1); (3) ΔG of hetero-dimer (kcal.mole-1)



(2)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bison schoetensacki voucher GAO1 mitochondrion, complete genome	52.0	52.0	100%	2e-04	100%	KU886087.1
Bison bonasus isolate Cc3/AF005 mitochondrion, complete genome	52.0	52.0	100%	2e-04	100%	KX553934.1
Bison bonasus isolate Cc2/22533 mitochondrion, complete genome	52.0	52.0	100%	2e-04	100%	KX553933.1
Bison bonasus isolate Bb3/BS607 mitochondrion, partial genome	52.0	52.0	100%	2e-04	100%	KX553932.1
Bison bonasus isolate Bb2/BS589 mitochondrion, complete genome	52.0	52.0	100%	2e-04	100%	KX553931.1
Bison bonasus isolate Bb1/BS587 mitochondrion, complete genome	52.0	52.0	100%	2e-04	100%	KX553930.1

(3)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bos taurus mitochondrial CYTB mRNA for cytochrome b, partial cds	58.0	58.0	100%	5e-06	100%	LC131290.1
Bos taurus isolate HT2_MLT10 haplogroup T3d mitochondrion, complete genome	58.0	58.0	100%	5e-06	100%	KT343749.1
Bos taurus isolate HT1_MLT04 haplogroup T3c mitochondrion, complete genome	58.0	58.0	100%	5e-06	100%	KT343748.1
Bos taurus haplotype H2 cytochrome b (cytb) gene, complete cds; mitochondrial	58.0	58.0	100%	5e-06	100%	KT151961.1
Bos taurus haplotype H1 cytochrome b (cytb) gene, complete cds; mitochondrial	58.0	58.0	100%	5e-06	100%	KT151960.1
Bos taurus isolate MACT mitochondrion, complete genome	58.0	58.0	100%	5e-06	100%	KU891849.1

(4)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sus scrofa isolate Vietnam_MBT4 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982660.1
Sus scrofa isolate Vietnam_BV02 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982659.1
Sus scrofa isolate Vietnam_BA30 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982658.1
Sus scrofa isolate Vietnam_BA24 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982657.1
Sus scrofa isolate Vietnam_BA12 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982655.1
Sus scrofa isolate Vietnam_BA9 mitochondrion, complete genome	54.0	54.0	100%	6e-05	100%	KX982654.1

Figure 1. (1) Pairing of F and R_P (left) and F and R_C (right) on *Cytochrome-b* sequences from pork and beef respectively (Genbank access codes: AB015081 and AY952955 respectively); all BLAST results of (2) F primer, (3) R_C primer and (4) R_P primer showed specificity

From the results in Table 2, it has been observed that physical values of length, % GC, melting point and difference in melting point were satisfactory in primer design. All ΔG was greater than -9kcal.mol^{-1}. The specificity tested by the BLAST and Annhyb program and shown in Fig. 1 described that the ability of pairing specifically on *Cytochrome-b* gene of pork and beef gained similarity levels of 100% and there was no presence of pairing on *Cytochrome-b* gene sequences of other animals. In addition, results of testing with ClustalX software indicated that pairs of primers were specific to *Cytochrome-b* gene sequences from pork and beef. Based on the results obtained, the pairs of primers were selected for use in subsequent experiments.

3.2. Construction of m-PCR procedure

Six experimental samples were extracted, including beef, pork, chicken, shrimp, fish and squid samples. These samples were extracted by the column method of Bioline, UK. The extraction products were checked their OD values and A260/A280 ratios.

Table 3

OD values and A260/A280 ratios

Beef	0,55	1,98	27,3
Pork	0,27	2,0	13,3
Chicken	2,38	2,0	119,1
Fish	0,4	2,05	19,9
Shrimp	0,21	1,98	10,3
Squid	0,42	2,02	21,1

The results (Tab. 3) showed that all A260/A280 values of the extracts in the range of 1.8 to 2.0, which suggested that DNA extraction products of these samples were not protein-infused.

Results of experimentally testing specificity of the primer

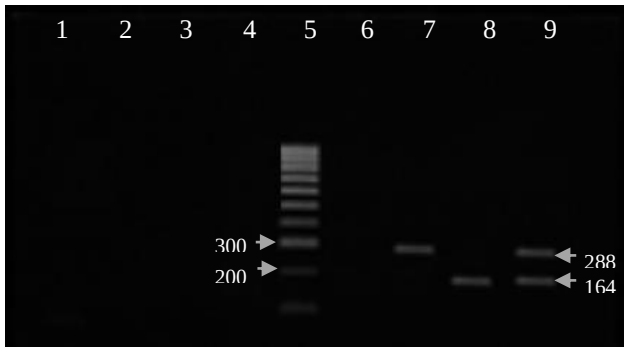


Figure 2. Results of experimentally testing specificity of the primer pairs

Well 1: distlled; Well 2: shrimp DNA; Well 3: fish DNA; Well 4: squid DNA; Well 5: Ladder DNA; Well 6: chicken DNA; Well 7: pork DNA; Well 8: beef DNA; Well 9: pork + beef DNA

The amplification results of researching specificity of the primer pairs, shown in Figure 2, described that there was the presence of product lines with expected size of 164bp for cow and of 288bp for pigs. It has been indicated that pairs of primers were specific to the *Cytochrome-b* gene sequence and could not amplify the target sequences in both theoretical and experimental samples of shrimp, fish, squid and chicken. To confirm this specificity, two PCR samples were sequenced at Nam Khoa Company, Vietnam.

The results of sequencing in Figure 3 described that product peaks were very clear except for the initial nucleotide sequence in which sequencing signal at the location of primers paired was not clear. However, the readable region (mid sequence) was very clear and completely specific when tested with BLAST. The BLAST results in Figure 4 showed that this sequence is highly homologous with the *Cytochrome-b gene* of beef (*Bos Taurus*) (access code: LC131290) with a similarity of 96%, E-value=8e-57, Ident=100% and of pork (Vietnam's sus scrofa) (access code: KX982660) with similarity of 98%, E-value=2e-121, Ident=99%.

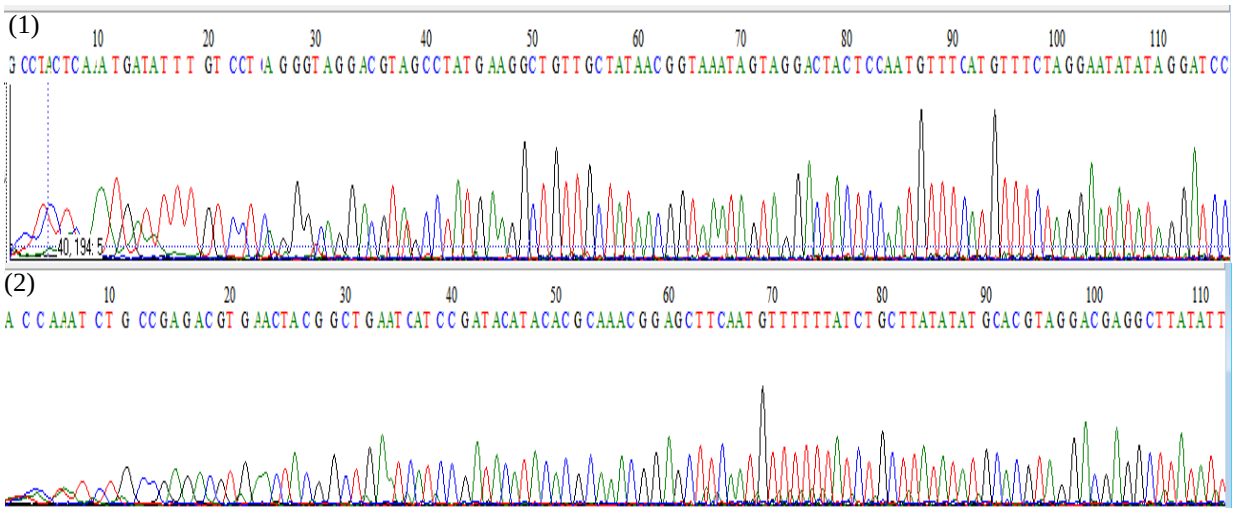


Figure 3. Results of sequencing (1) the reverse (pork primers) of the PCR product of pork sample (2) the reverse (beef primers) of the PCR product of beef sample

(1) Description	Max score	Total score	Query cover	E value	Ident	Accession
Bos taurus mitochondrial CYTB mRNA for cytochrome b, partial cds	230	230	96%	8e-57	100%	LC131290.1
Bos taurus isolate HT2_MLT10 haplogroup T3d mitochondrion, complete genome	230	230	96%	8e-57	100%	KT343749.1
Bos taurus isolate HT1_MLT04 haplogroup T3c mitochondrion, complete genome	230	230	96%	8e-57	100%	KT343748.1
Bos taurus haplotype H2 cytochrome b (cytb) gene, complete cds; mitochondrial	230	230	96%	8e-57	100%	KT151961.1
Bos taurus haplotype H1 cytochrome b (cytb) gene, complete cds; mitochondrial	230	230	96%	8e-57	100%	KT151960.1
Bos taurus isolate MACT mitochondrion, complete genome	230	230	96%	8e-57	100%	KU891849.1
Bos taurus haplogroup Q1 mitochondrion, complete genome	230	230	96%	8e-57	100%	KP637147.1
Bos taurus cytochrome b (cytb) gene, partial cds; mitochondrial	230	230	96%	8e-57	100%	KT946968.1
Bos taurus isolate 36 cytochrome b (cyt b) gene, partial cds; mitochondrial	230	230	96%	8e-57	100%	KT260196.1
Bos taurus isolate 35 cytochrome b (cyt b) gene, partial cds; mitochondrial	230	230	96%	8e-57	100%	KT260195.1

(2) Description	Max score	Total score	Query cover	E value	Ident	Accession
Sus scrofa isolate Vietnam_MBT4 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982660.1
Sus scrofa isolate Vietnam_BV02 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982659.1
Sus scrofa isolate Vietnam_BA30 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982658.1
Sus scrofa isolate Vietnam_BA24 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982657.1
Sus scrofa isolate Vietnam_BA19 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982656.1
Sus scrofa isolate Vietnam_BA12 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982655.1
Sus scrofa isolate Vietnam_BA9 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982654.1
Sus scrofa isolate Vietnam_BA7 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982653.1
Sus scrofa isolate Vietnam_MCN16 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982652.1
Sus scrofa isolate Vietnam_MCC09 mitochondrion, complete genome	446	446	98%	2e-121	99%	KX982651.1

Figure 4. BLAST results of (1) PCR product sequence of beef sample-R_C primer; (2) PCR product sequence of pork sample-R_P primer

From here, we optimized two parameters including concentration of the primers and hybrid temperature of the m-PCR reaction. Results of the surveys determined that the hybrid temperature for detection of pork and beef by m-PCR was 59^0_C and the concentration of each primer was 0.4µM. (data not given).

Sensitivity of the m-PCR method

The sensitivity of m-PCR was determined by using a mixture of two pork and beef DNAs with the concentration of 10ng/µl, diluted 10-fold to 0,001ng/l. The results in Well 4 of Figure 5 showed that the positive signal was stable at 0.1ng/µl. This result also coincided with the results of previously published studies (Haining He et al, 2015; Abdul – Hanssan et al, 2014). Therefore, the use of 3µl DNA extraction for m-PCR determined the sensitivity of m-PCR to be 0.1ng/µl.

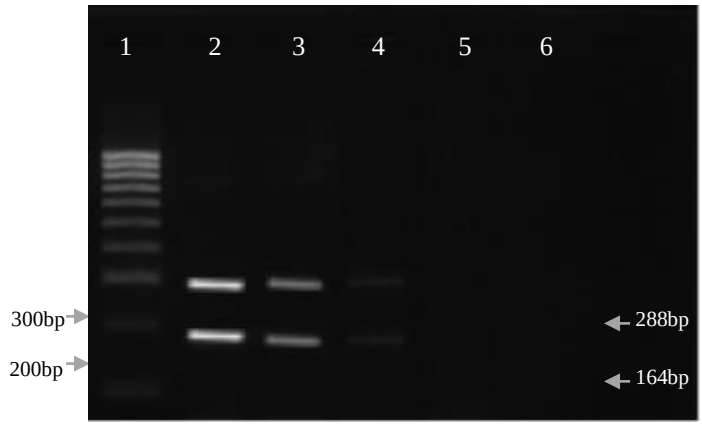


Figure 5. Results of m-PCR from a mixture of 2 pork and beef DNAs with different concentrations

Well 1: Ladder 100 bp; Well 2: 10ng/µl; Well 3: 1ng/µl; Well 4: 0,1ng/µl; Well 5: 0,01ng/µl; Well 6: 0,001ng/µl

Based on theoretical and experimental results, it is suggested that the study has been successfully initiated the development of the *Cytochrome-b* gene amplification protocol for the detection of pork present in food products processed from beef.

Results of testing m-PCR procedure on actual sample

After successfully building the experimental protocol, the first trials were performed on 24 samples of beef sausage purchased from shops and supermarkets in Ho Chi Minh City with the ingredients on the packages containing no pork. The results on the electrophoresis board were presented in Figure 6.

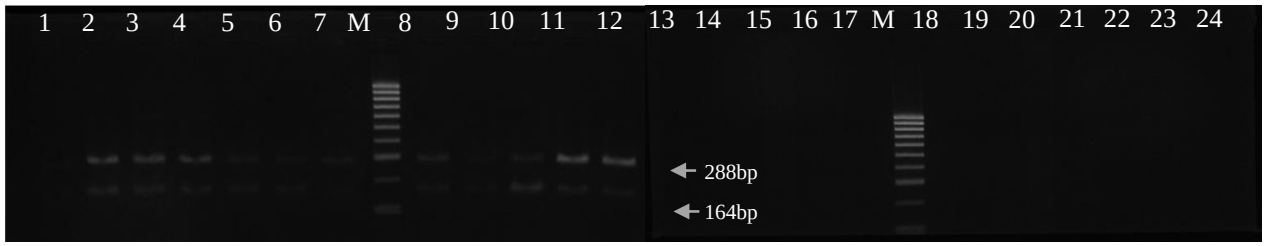


Figure 6. Results of detecting presence of pork in the beef sausage samples
Well 1-12: A manufacturer; Well 13-24: B manufacturer; M: Ladder

The results in Figure 6 showed that most of the beef sausage samples from A manufacturer, 11/12 of samples (well 2-12) appear 2 bands with size of 164bp to cow and 288bp to pig, it means 11 samples of beef sausage stated above are positive to pork, 1/12 of sample (well 1) no beef and pork, all beef sausage samples from B manufacturer, 12/12

of samples (well 13-24), even contained neither pork nor beef, while both of them listed only beef on the packages. The uneven brightness in the infected samples is due to the presence of animal protein in different samples, the different concentrations of DNA extracted from different samples, which resulted in amplifications with different luminosity of electrophoresis bands.

4. Conclusion

The m-PCR protocol was developed to detect pork and beef, in which one common pair of primers was used to detect both pork and beef while reverse primers were distinct for each species. The study indicated that the primers in pork and beef were specific, the hybrid temperature between the primer and the DNA template was 59°C, the concentration of each primer was 0.4µM, the lowest concentration of pork, beef DNAs detected by m-PCR was 0,1ng/µl. Through surveying 24 samples of beef sausages, the result shows m-PCR technique can develop one quick check method to detect pork in the food products processed from beef■

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