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Detection and quantification of pork and rat DNA in processed meats using multiplex quantitative Real-Time PCR (m-qPCR)

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ABSTRACT In addition to the issue of pork contamination, processed meats frequently contain traces of rat meat. Therefore, detection and quantification of the pork and rat DNA in cases of meat and processed meat adulteration are necessary. In the current study, two gene targets of the *cytochrome b* for pigs and the *Mt-atp6* of *Rattus norvegicus* for rats were used in the absolute multiplex quantitative real-time PCR (m-qPCR). The sample DNA was amplified with a standard as positive control in the various concentration of 1000 pg, 100 pg, 10 pg, 0.1 pg, 0.01 pg, and 0.001 pg. There were 25 processed meat samples and 5 fresh meat samples identified in this study. Among the total of 30 samples assessed, 6 samples were successfully detected and quantified their pork and rat DNA contamination. One sample was contaminated with pork DNA with a concentration of 2.451×10^{-4} pg ("Meatball 3). Five samples were contaminated with rat DNA with a concentration of 3.603×10^{-11} pg ("Sempol 3"), 2.196×10^{-10}pg ("Meatball 6"), 4.908×10^{-11} pg ("Siomay 3"), 1.489×10^{-10} pg ("Grinding 2"), and 3.564×10^{-10} pg ("Grinding 4"). In this study, we have discovered that the contamination of pork and rat were detected in the samples. It suggested that this method is applicable for detecting the contaminant in processed meat samples.

KEYWORDS *Cytochrome b*; Multiplex PCR; *Mt-atp6*; Processed meat; qPCR

1. Introduction

Halal food is essential for the Indonesian Muslim community. As the global halal food market is growing, however, there are increased risks of fraud and adulteration. It has been reported that frequent incidents of meat fraud and adulteration occurred in ASEAN countries over 20 years (2000-2020) (Owolabi and Olayinka 2021). Indonesia itself also face high case of meat adulterations. They were mostly formalin meat, "glonggong" meat (a meat with water injections), rotten meat, and fake meat or species substitution (Ramli et al. 2018). In the case of species substitution, Indonesia has also faced numerous cases of meat and processed meat being mixed with pork (Sari 2017; Maulani et al. 2020; Nida et al. 2020; Mustaqimah et al. 2021; Siswara et al. 2022; Waluyo et al. 2023). Recently, many cases of adulterated meat with rats have also been discovered and the numbers are increased (Suryawan et al. 2020; Lestari et al. 2022).

A halal product regulation of Law Number 33 of 2014 concerning Halal Product Certification, amended by Law Number 11 of 2020 concerning Job Creation, is being implemented in Indonesia. The law demands that all products must undergo halal standard and certification. They have to be labelled as halal; thus, it has to be made using halal ingredients. These regulations have implications for legal consequences if they are not complied. Therefore, the regulation should strongly protect meat ingredients and their derivatives from adulteration. Despite the presence of this regulation, the practice of mixing a specific type of meat with meat from a different species often occurs to increase profits in certain industries. Meat adulteration is considered a crime as it introduces unsafe and low-quality products into the market. Such cases happen when meat is mixed with cheaper meats with similar characteristics, such as pork, rat, and a combination of pork and rat. Consequently, meat detection methods are vital for detection of such contamination. The meat detection can provide halal authenticity as well as to prevent and decrease meat and processed meat adulteration cases in the market.

The most accurate method to detect meat adulteration is based on the DNA marker using polymerase chain reaction (PCR) (Tanabe et al. 2007b; Sari 2017; Maulani et al. 2020; Waluyo et al. 2023; Mustaqimah et al. 2021).

PCR was selected because this technique has high sensitivity and can amplify the specific targets, even those of highly complex genomic sequences (Tanabe et al. 2007a). Many studies have used PCR to detect pork contamination in meat and processed meats. The specific gene target for pork detection is the porcine cytochrome *b* region of mitochondrial DNA (Tanabe et al. 2007b,a). Other gene targets commonly used are the genes of *ND2*, *ND5*, and *12S* rRNA (Chisholm et al. 2005; Kesmen et al. 2009; Cahyadi et al. 2020). However, with the rising number of rat contamination, there has also been an increase in studies focusing on rat detection. The genetic marker for rat detection is the *Mt-atp6* of *Rattus norvegicus* gene (Sihotang et al. 2023). Other gene targets for rat detection in meat products are *ND5*, *cytb 42*, and *mt-CoI* genes (Widyasari et al. 2015; Sihotang et al. 2021; Masnaini et al. 2023). Two protein markers of *Rattus norvegicus* have also been reported to be used for rat detection in meat produced from non-halal slaughter (Aini et al. 2022). As the complexity of meat adulteration cases are increasing lately, thus a particular detection is needed to overcome these complex adulteration cases. Multiplex quantitative Real-time PCR (m-qPCR) is an evolution of PCR detection method, which not only can detect but also quantify the contamination. However, research on the detection and quantification of pork and rats simultaneously in meat and processed meat are yet limited.

Simultaneous detection of multiple species contamination in meat and processed meat can be conducted using multiplex PCR. The multiplex PCR uses several primers simultaneously in one reaction to amplify multiple target sequences (Indriati and Yuniarsih 2019). It has been reported that multiplex PCR assay can be used to detect species substitutions of goat, cattle, chicken, and pig (Cahyadi et al. 2021). Multiplex PCR has been reported to discriminate the presence of beef and pork in meat samples using the *cytb* gene as the marker (Indriati and Yuniarsih 2019). It is described that the primers of the *cytb* gene can produce different lengths of DNA fragments based on the specific length of each species; therefore, it is utilized to discriminate two species simultaneously (Indriati and Yuniarsih 2019). Therefore, this study aims to identify pork and rat contamination in meat and processed meat using two pairs of primers derived from porcine *cytb* sequences and *Mt-atp6* of *R. novergicus* gene sequences using multiplex PCR. Furthermore, the quantifications of pork and rat contaminations in the samples were performed in this study.

2. Materials and Methods

2.1. Sample collection

The samples in this study were taken from the East Jakarta region, as previous research had found rat DNA contamination in sausage samples from the street vendors in the East Jakarta area. The samples identified were processed meat obtained randomly from night market traders in East Jakarta and raw meat from meat grinding locations in East Jakarta. The samples consisted of 25 types of processed meat, including 7 sempols, 6 siomay, 7 meatballs, and 5 dimsum. Meanwhile, 5 samples of raw meat were collected from different grinding shops in East Jakarta. The identified samples were uncertified halal processed meats.

2.2. DNA extraction

DNA extraction began with sample preparation. The kit used in this method was the gSYNCTM DNA Extraction kit (Geneaid, Taiwan). It comprised GST buffer, proteinase K, GBS buffer, GD columns, W1 buffer, wash buffer, and elution buffer. A 25 mg sample was meticulously weighed and placed in a 1.5 mL microtube, added with 200 µL of GST buffer and 20 µL of proteinase K, vortexed for 15 s, and incubated overnight at 60 °C. After incubation, the samples were centrifuged for 2 min at 12,000 rpm. An amount of 200 µL of the supernatant was pipetted and then put into a 1.5 mL microtube. 200 µL of GSB buffer was added to the tube and then vortexed for 10 s. An amount of 200 µL of absolute ethanol was put to the tube and then vortexed for 10 s. The sample solution was pipetted and then transferred to the GD column. The tube was centrifuged for 1 min at 12,000 rpm. The GS column was filled with 400 µL of W1 buffer, and it was centrifuged for 30 s at 12,000 rpm. The supernatant was discarded, and the remaining part was filled with 600 µL of wash buffer, and it was then centrifuged for 30 s at 12,000 rpm. The supernatant was discarded. After the centrifugation of GS column for 3 min at 12,000 rpm, the GS column was transferred into a new 1.5 mL microtube. The GS column was filled with 50 µL of elution buffer and incubated at room temperature for 3 min. At 12,000 rpm, the GS Column tube was centrifuged for 30 s. The DNA was characterized using a spectrophotometer (Thermo Scientific^{TM} NanoDrop One). DNA amplification was conducted using the qPCR method.

2.3. Multiplex-PCR

The results of DNA extraction were amplified using the Toyobo THUNDERBRID qPCR probe targeting the *cytb* gene for pigs (Tanabe et al. 2007a) and the *Mt-atp6* gene *R. norvegicus* for rats (Sihotang et al. 2023). The 20 µL total PCR reaction included 2 µL of DNA template, 0.6 µL of primers, 0.4 µL of probe, and 10 µL of Toyobo THUNDERBRID qPCR probe. Up until 20 µL of nuclease-free water (NFW) was supplied, the reaction volume remained maintained. Using a CFX96 Touch Deep Well Real-Time PCR, DNA was amplified for 45 cycles: denaturation at 95 °C for 15 s, annealing at 57 °C for 30 s, and extension at 60 °C for 30 s. The initial denaturation temperature was set for 1 min. For the *cytb* gene (pigs) and the *Mt-atp6* gene (rats), there were two probes used in this amplification: one tagged with fluorescent HEX (rats) and the other with fluorescent FAM (pigs). The segment sequences of pig and rat primer were used as a standard, as shown in Table 1. There were seven standards at concentrations of 1000 pg, 100 pg, 10 pg, 1 pg, 0.1 pg, 0.01 pg, and 0.001

TABLE 1 Sequences of primers and probes.

Targets		Sequence
Pork	Forward Primer	5'- CTTGCAAATCCTAACAGGCCTG -3'
(Porcine DNA)	Reverse Primer	5'- CGTTTGCATGTAGATAGCGAATAAC -3'
	TaqMan MGB Probe	5'-(FAM)-ACAGCTTTCTCATCAGTTAC-(NFQ)(MGB) -3'
	RnATP6-161 Forward	5'-ACACCAAAAGGACGAACCTG -3'
Rat (*Mt-atp6 Rattus norvegicus* gene)	RnATP6-161 Reverse	5'-AGAATTACGGCTCCTGCTCA -3'
	RnATP6-161 Probe	5'- [HEX]-TTCTAGGGCTTCTTCCCCAT-[QSY] -3'

pg.

3. Results and Discussion

The result of the concentration of the DNA sample was in the range of 7.7 – 148.2 ng/ µL, as shown in Table 2. The analysis showed that the lowest concentration was found in the meatball sample at 7.7 ng/ µL, while the highest was found in the meat sample at 148.2 ng/ µL. The DNA purity of the samples ranged between 1.82 – 2.06.

TABLE 2 Concentration and purity of DNA samples.

No	Sample Code	Concentration (ng/µL)	Purity (260/280 nm)
1	Sempol 1	57.2	1.84
2	Sempol 2	39.9	1.84
3	Sempol 3	11.5	1.98
4	Sempol 4	51.2	2.00
5	Sempol 5	20.0	2.00
6	Sempol 6	13.9	1.91
7	Sempol 7	22.2	2.00
8	Meatball 1	7.8	1.95
9	Meatball 2	98.4	2.01
10	Meatball 3	28.1	2.06
11	Meatball 4	36.5	2.01
12	Meatball 5	7.7	2.06
13	Meatball 6	75.2	2.03
14	Meatball 7	28.7	1.97
15	Siomay 1	59.7	1.82
16	Siomay 2	63.5	1.93
17	Siomay 3	13.9	1.90
18	Siomay 4	17.1	1.86
19	Siomay 5	20.5	1.87
20	Siomay 6	30.9	1.95
21	Dimsum 1	124.2	2.01
22	Dimsum 2	132.6	1.98
23	Dimsum 3	73.9	2.00
24	Dimsum 4	24.5	1.99
25	Dimsum 5	77.7	1.89
26	Grinding 1	110.1	2.06
27	Grinding 2	115.8	2.06
28	Grinding 3	100.7	2.01
29	Grinding 4	148.2	2.05
30	Grinding 5	120.1	1.98

In this study, 6 of the total 30 samples assessed were contaminated with pork and rat DNA. The DNA samples were amplified using the method of absolute multiplex quantification real-time PCR (m-qPCR). The amplification results were presented as a standard curve for the *cytb* gene (pigs) in Figure 1, a standard curve for the *Mt-atp6* gene (rats) in Figure 2, and the standard concentrations in Table 3. The amplification results of DNA samples are presented in Figure 3 and Table 4. The samples in this study include seven sempol, seven meatballs, six siomay, five dimsums, and meat from several grinding locations. Of the 30 samples, one sample was positive for pork ("Meatball 3") and five were positive for rats ("Sempol 3, Siomay 3, Meatball 6, Grinding 2, and Grinding 4").

3.1. Discussion

Cases of processed meat contaminated with other types of meat have occurred in Indonesia; therefore, appropriate methods are required to identify meat contamination. DNA-based methods, i.e., conventional PCR, real-time PCR, and qPCR, are often used to identify meat contaminations (pork and rats) (Chisholm et al. 2005; Kesmen et al. 2009; Widyasari et al. 2015; Indriati and Yuniarsih 2019; Salamah et al. 2019; Cahyadi et al. 2020, 2021; Sunaryo et al. 2022). Addition to that, a method for detecting substitutions of several species simultaneously is also necessary (Indriati and Yuniarsih 2019; Cahyadi et al. 2020, 2021). Pork and rat were chosen in this study as they are common contamination found in the meat adulteration cases in Indonesia. Here in this study, we used a multiplex quantitative Real-Time PCR (m-qPCR) as a simultaneous detection method. It can detect multiple species at one time as current adulteration cases in Indonesia have a high probability of multiple species substitution. This method enhances efficiency by shortening the detection time of several species at once. This method has also not been widely explored on the meat substitution cases in Indonesia.

In the current investigation, our absolute multiplex quantitative real-time PCR (m-qPCR) has successfully proven to identify and quantify species substitutions in the meat and processed meat products simultaneously. The method used consist of two main steps, which are DNA

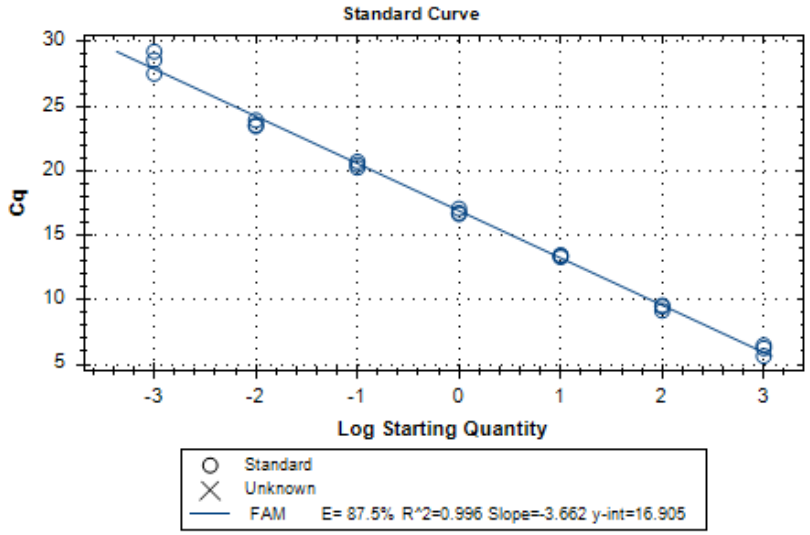

FIGURE 1 Standard Curve of *Cytochrome B* Gene (Pig).

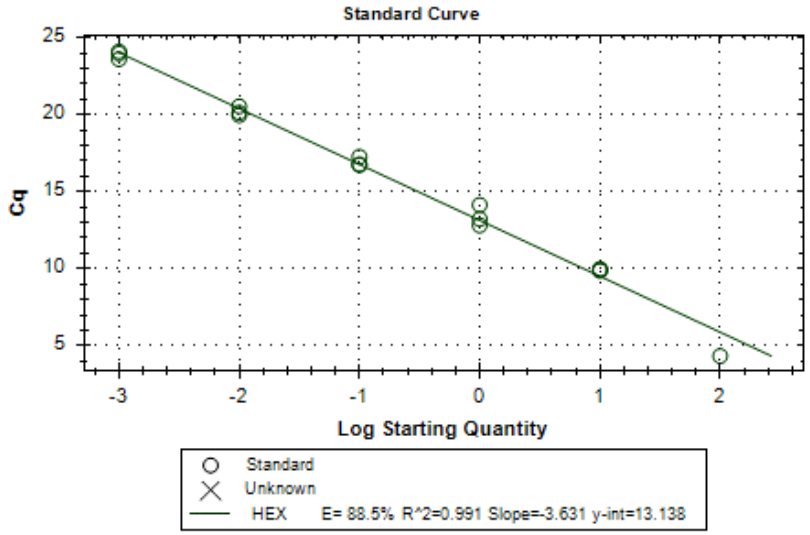

FIGURE 2 Standard Curve of *Mt-atp6* Gene (Rat).

extraction and DNA amplification stages. A spin column-based extraction kit was performed to extract the genomic DNA of the samples. The concentration produced using this method was 7.7–148.2 ng/µL. The DNA purity was 1.82–2.06, which meets the specification of 1.7–2.0 (Adriany et al. 2020; Sunaryo et al. 2023). In this study, the spin column method was used as it produces the purer DNA than other methods (Andalia et al. 2023). It is known that the DNA yields from DNA extraction kit is usually lower and the purity is higher (Liao et al. 2017). The re-

TABLE 3 Cq value and standard concentration.

No	Standard (Std)	Cq FAM (Babi)	Concentration FAM (Babi)	Cq HEX (Tikus)	Concentration HEX (Tikus)
1	Std-1	4.93	1,00E+06	4.02	1,00E+06
2	Std-2	9.63	1,00E+05	7.72	1,00E+05
3	Std-3	13.09	1,00E+04	12.35	1,00E+04
4	Std-4	15.59	1,00E+03	13.88	1,00E+03
5	Std-5	17.31	1,00E+02	16.82	1,00E+02
6	Std-6	20.81	1,00E+01	19.38	1,00E+01
7	Std-7	22.49	1,00E+00	22.31	1,00E+00

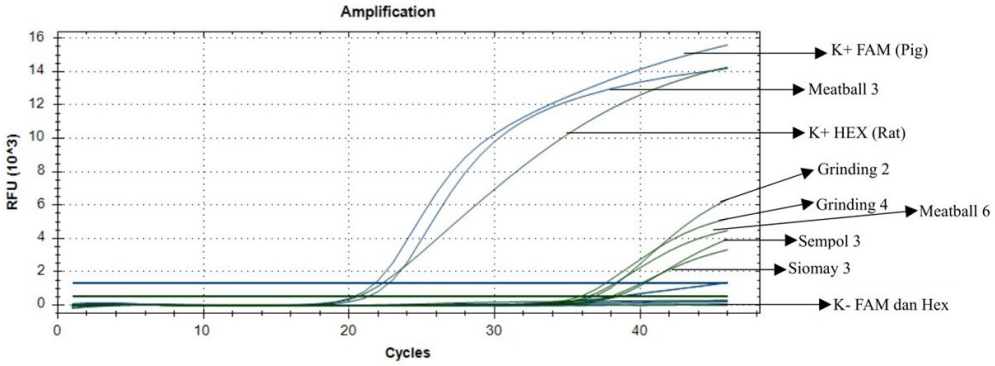

FIGURE 3 Result of DNA Sample Amplification.

sults in this study are also in line with that finding that the DNA concentration obtained in this study is also considered lower, however the purity is considered higher. The higher purity provided high-sensitivity detection methods.

Further, the DNA amplification method is the absolute quantification method (qPCR). In this method, the sample DNA is amplified with a positive control in the form of a standard. The standard consists of a combination of pork and rat DNA segment sequences with various concentrations, as shown in Table 1. Those DNA segment sequences

TABLE 4 Cq value and DNA sample concentration.

No	Sample Code	Cq FAM (Babi)	Concentration FAM (Babi)	Cq HEX (Tikus)	Concentration HEX (Tikus)
1	Sempol 1	N/A	N/A	N/A	N/A
2	Sempol 2	N/A	N/A	N/A	N/A
3	Sempol 3	N/A	N/A	38.37	3.603E-11
4	Sempol 4	N/A	N/A	N/A	N/A
5	Sempol 5	N/A	N/A	N/A	N/A
6	Sempol 6	N/A	N/A	N/A	N/A
7	Sempol 7	N/A	N/A	N/A	N/A
8	Meatball 1	N/A	N/A	N/A	N/A
9	Meatball 2	N/A	N/A	N/A	N/A
10	Meatball 3	22.69	2.451E-04	N/A	N/A
11	Meatball 4	N/A	N/A	N/A	N/A
12	Meatball 5	N/A	N/A	N/A	N/A
13	Meatball 6	N/A	N/A	36.47	2.196E-10
14	Meatball 7	N/A	N/A	N/A	N/A
15	Siomay 1	N/A	N/A	N/A	N/A
16	Siomay 2	N/A	N/A	N/A	N/A
17	Siomay 3	N/A	N/A	38.04	4.908E-11
18	Siomay 4	N/A	N/A	N/A	N/A
19	Siomay 5	N/A	N/A	N/A	N/A
20	Siomay 6	N/A	N/A	N/A	N/A
21	Dimsum 1	N/A	N/A	N/A	N/A
22	Dimsum 2	N/A	N/A	N/A	N/A
23	Dimsum 3	N/A	N/A	N/A	N/A
24	Dimsum 4	N/A	N/A	N/A	N/A
25	Dimsum 5	N/A	N/A	N/A	N/A
26	Grinding 1	N/A	N/A	N/A	N/A
27	Grinding 2	N/A	N/A	36.88	1.489E-10
28	Grinding 3	N/A	N/A	N/A	N/A
29	Grinding 4	N/A	N/A	35.96	3.564E-10
30	Grinding 5	N/A	N/A	N/A	N/A

targeted the mitochondrial DNA, in which mitochondrial DNA is commonly used to identify the species (Liao et al. 2017). The amplification resulted in two standard curves: the pig standard (FAM) in Figure 1 and the rat standard (HEX) in Figure 2. The standard curve provides information regarding reaction performance with various parameters, namely efficiency (E), R^2, and slope.

The pig standard curve (FAM) shows the values of E at 87.5%, R^2 at 0.996, and slope at -3.662, while the rat standard curve (HEX) shows the values of E at 88.5%, R^2 at 0.991, and slope at -3.631. The 87.5% and 88.5% efficiency values indicate the relatively efficient qPCR amplification reactions. For the multiplex PCR, it is known that the amplification efficiency should be in the range of 90–110% (quantitative) and 80–120% (qualitative) (Broeders et al. 2014). Those broader range allow the reproducibility of amplification (Broeders et al. 2014). Even though the values are slightly below the ideal criteria of 90% – 110%, the values of 87.5% and 88.5% are still considered reasonable and can provide reliable results. The linearity of R^2 value ≥ 0.98 represents the ideal linearity for multiplex PCR (Broeders et al. 2014). The R^2 values of 0.996 and 0.991 obtained in our study indicated that the amplification data is strongly correlated with the linear model on the standard curve. It described that the relationship between the logarithm of the initial amount of target DNA and the fluorescence uptake is linear. The slope values of -3.662 and -3.631 from our study suggested a successful amplification and indicate a relatively good efficiency level (Luque-Perez et al. 2013; Tan et al. 2020; Mariyani et al. 2021).

Of the 30 samples identified, one contained pig DNA, and five were positive for rat contamination. The sample that contained pig DNA was ”Meatball 3” with a concentration of 2.451×10^{-4} pg and a Cq value of 22.69. Irwandi et al. (2020) reported the presence of pig DNA contaminations in meatballs, where two out of three samples tested positive for pig DNA. Similarly, Purwantoro et al. (2022) detected pig DNA contamination in sausage samples, with one out of five samples showing the presence of pig DNA. Our result is in line with that of Cahyadi et al. (2020) revealing how multiplex PCR used to detect multiple species contamination in one reaction.

Samples contaminated with rats were ”Sempol 3” with a concentration of 3.603×10^{-11} pg and a Cq value of 38.37, ”Meatball 6” with a concentration of 2.196×10^{-10} pg and a Cq value of 36.47, ”Siomay 3” with a concentration of 4.908×10^{-11} pg and a Cq value of 38.04, ”Grinding 2” with a concentration of 1.489×10^{-10} pg and a Cq value of 36.88, and ”Grinding 4” with a concentration of 3.564×10^{-10} pg and a Cq value of 35.96. Sunaryo et al. (2022) also reported rat contamination in processed products, with one out of 30 sausage samples contaminated with rat DNA. Meanwhile, Susilowati (2019) discovered cases of non-halal meat contamination in grinding locations, with five out of 30 meat samples found to be contaminated with pork.

4. Conclusions

In this study, the multiplex quantitative Real-Time PCR (m-qPCR) brings a significant result in successfully detect and quantify the contaminated samples. Of the 30 samples, one contained pork DNA, and five were positive for rat contamination. The sample contaminated with pig DNA was ”Meatball 3”. Meanwhile, the samples contaminated with rats were ”Sempol 3”, ”Meatball 6”, ”Siomay 3”, ”Grinding 2”, and “Grinding 4”. The method serves as an effective technique for analyzing and detecting multiple species substitution at once. Furthermore, the multiplex quantitative Real-Time PCR (m-qPCR) can be widely used to assess, trace, and calculate the contamination for ensuring food quality and detecting complex food adulteration.

Acknowledgments

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Authors' contributions

NAN and EDP designed the study. NAN carried out the laboratory work. NAN and EDP analyzed the data. EDP wrote the manuscript. All authors read and approved the final version of the manuscript

Competing interests

The author declared that there were no conflicts of interest in this study.

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