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Simultaneous identification of four meat species (cattle, chicken, fish, and pig) using next generation sequencing (NGS)

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Abstract

Meat adulteration has become a serious problem in global which directly affects to food consumers and producers. Therefore, it requires a tool to authenticate meat species to ensure safety of food products. Next generation sequencing (NGS) coupled with ribosomal RNA mitochondrial DNA gene can be used to analyze mixture of meat species in multiple meat samples. Therefore, this study aims to utilize NGS coupled with rRNA gene to identify 4 meat species (cattle, chicken, fish, and pig). Three primer sets (12S-Ki, 16S-KH, and 16S-Ki) were used to amplify DNA from the four meat species. All primer sets could be successfully amplified DNA fragments which corresponded to their size expectation. 16S-KH showed better detection effect in all species comparing with others. While the 12S-Ki and 16S-Ki could not be used to amplify in fish and chicken species. This may occur due to mismatches between sequences of primers and annealed regions of these species. Library construction of all PCR amplicons were prepared and sequenced by NGS. Amplicons amplified by 12S-Ki (fish) and 16SKi (chicken and fish) could not be mapped to the database because no PCR amplicons could not be amplified. NGS coupled with 16S-KH was then evaluated for precision test. The experimental precision was directly investigated comparing the results obtained from libraries that derives from DNA of four meat species which separately amplified for 3 different runs. As expected, the number and proportion of mapped reads between three different runs were also concordant. The percentage of mapped reads ranged from 14.05% to 31.04%, 15.14% to 31.98%, and 14.21% to 33.05% (1st, 2nd, and 3rd run, respectively). This demonstrated that NGS coupled with rRNA mtDNA gene could be reliably implemented as a routine testing. This developed technique can be applied to control and monitor meat adulterations in halal meat production and industry.

Keywords: *Next Generation Sequencing, Ion Torrent PGM, Halal species, meat species identification, ribosomal RNA*

INTRODUCTION

Meat adulteration has become a serious predicament in global. The adulterations could be occurred by accidental contamination or mislabelling. The cheaper meat is often used to substitute in raw meat and meat products such as beef meatballs substituted with rat meat in

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Indonesia (Suryawan et al., 2020), mutton adulterated by rat meat in China (Fang & Zhang, 2016), and chicken substituted with pork in China (Yin et al., 2020). Recently, fraudulent labelling of meat product was found in Thailand. Pork dyed with cow blood was sold as beef in the fresh markets (Denyingyhot et al., 2022). This directly affected to halal entrepreneurs, SMEs and restaurants which operate their business for using raw meats as ingredients. In addition, this raises concerns about food safety, religions, and ethics. Therefore, it requires a tool to identify meat species for food authentication.

Next generation sequencing (NGS) is an advanced high throughput sequencing technology which provides a massively parallel analysis in multiple samples. It has a high potential to use as tool for routine testing of meat inspection and authentication. Recently, many disclosed studies have reported in seafood (Giusti et al., 2017; Piredda et al., 2022), dairy products (Ribani et al., 2018), poultry meat (Dobrovolny et al., 2019), and pork (Akbar et al., 2021). Therefore, this emerging technology will provide the reliable tool for identifying the mixed meat species in meat product without any previous knowledge required. It will help the food producers to assure the authenticity of meat species with precise labeling on the products.

LITERATURE REVIEW

Thailand has a potential to serve halal food market in global with high volumes of halal meat production. However, obstacle in the meat production is adulteration of cheaper meat which prohibited by Islamic law. Halal meat product is commonly used beef and chicken which slaughtered following Islamic law and practices for consumption. Besides, fish is traditional allowed to use in Halal consumption and industrial usage. Pork is the most popular use in meat products. It is considered as the cheapest meat and widely used as a substitute for expensive meat such as beef (Denyingyhot et al., 2022). However, it is prohibited for Muslim consumer. Therefore, routine inspection of the prohibited meat adulteration is required.

Species identification is essential to determine mixed species in food and meat products. Many different methods have been used to identify the species of meat. Amongst, DNA techniques have been effectively developed and used in the meat species identification. (Amaral, 2021) Most of them employ Polymerase Chain Reaction (PCR) to amplify specific DNA fragments which further analyzed by different methods such as DNA barcoding (Kappel et al., 2017; Pan et al., 2020), real time PCR (Mahama et al., 2020), multiplex high-resolution melting analysis (Denyingyhot et al., 2021), and DNA strip (Denyinghot et al., 2022). Commonly, ribosomal RNA mitochondrial DNA gene is used as targeting region for identifying the species of meat. There are hundreds to thousands of copies of mtDNA in each cell so it will lower risk to fail with degraded templates (Yang et al., 2014; Liu et al., 2021; Bertolini et

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al., 2015). The traditional workflow analysis is to amplify the complete specific gene sequence by PCR and then use the conventional Sanger sequencing method for gene sequence analysis. However, this method can only sequence a single DNA fragment at the time. Therefore, it can not be applied for detecting mixtures of unknown species in meat products (Kappel et al., 2017).

Next generation sequencing (NGS) technology can be used to analyze meat products containing different species mixtures by parallel sequencing from a sample (Jagadeesan et al., 2019). In the last decade, NGS has transformed from being solely a research tool to becoming routinely applied in many fields including diagnostics, outbreak investigations, antimicrobial resistance, forensics, and food microbiology (Allard et al., 2018; Goodwin et al., 2016; Quainoo et al., 2017). The technology is developing at a rapid pace, with continuous improvement in quality and cost reduction and is having a major influence on food authenticity. The NGS coupled with 12S and 16S rRNA mitochondrial DNA gene have been successfully employed for identifying species of meat products. The species mixture of common meat (pig, cattle, sheep, chicken, turkey, duck, horse, rat, and rabbit) can be mixed and detected (Karlsson et al., 2007; Kitano et al., 2007; Giusti, 2017; Bertolini et al., 2015; Liu et al., 2021). Therefore, this proves that NGS coupled with rRNA mitochondrial DNA gene can be used as a tool for species identification. However, there is no reported study in fish species with these primer sets. Thus, this study aims to utilize NGS coupled with ribosomal RNA gene to identify 4 meat species including Halal meat species (cattle, chicken, and fish) and non-Halal meat species (pig).

RESEARCH METHODOLOGY

Sample

A total of four meat samples; cattle *(Bos indicus),* chicken (*Gallus gallus*), fish *(Chitala ornate)*, pig *(Sus scrofa)*, was obtained from purchased from supermarket in Bangkok during June 2020. They were transferred to the refrigerator to the laboratory and stored in -20˚C until use.

DNA extraction

DNA was extracted from 80-100 mg of meat sample using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The extracted DNA purity and quality was measured by NanoDrop™ 2000/2000c spectrophotometers (Thermo Fischer Scientific, MA, US). Calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm. Degradation of DNA of each species was checked by 1% agarose gel electrophoresis.

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PCR amplification

PCR amplification with the 12S rRNA and 16S rRNA universal primer pairs were performed in a volume of 50 µl containing 50 ng of the extracted DNA, 2x PCRBIO HS Tag Mix Red (PCR Biosystems Ltd., UK), 0.6 µM concentration of each universal primer (Table 1.). PCR was amplified on an Applied Biosystems Veriti™ 96 well Thermal Cycler (Thermo Fisher Scientific) with the following cycling program: denaturation at 95°C for 1 min; 32 cycles at 94°C for 15 s, 59°C, 63°C, and 62°C (12S-Ki, 16S-KH, and 16S-Ki, respectively) for 15 s, and 72°C for 30 s; final extension at 72°C for 7 min. The presences of PCR products were confirmed by 2% agarose gel electrophoresis.

Table 1. The sequences of universal primer.

Gene	Primer name	Primer Sequence (5' – 3')	Expected size (bp)	References
12S-Ki	Forward	CCCAAACTGGGATTAGATACCC	215-222	Karlsson et al., 2007
	Reverse	GTTTGCTGAAGATGGCGGTA		
16S-KH	Forward	GACGAGAAGACCCTATGGAGC	112-130	Karlsson et al., 2007
	Reverse	TCCGAGGTCGCCCCAACC		
16S-Ki	Forward	GCCTGTTTACCAAAAACATCAC	243-249	Kitano et al., 2007
	Reverse	CTCCATAGGGTCTTCTCGTCTT		

Library preparation, quantification, emulsion PCR, and sequencing

Preparation of barcoded libraries; A specific barcoded library was prepared for the amplicon obtained from each meat samples using the Ion Plus Fragment Library Kit (IPFL kit-Thermo Fisher Scientific), that allowed amplicons' end-repair and ligation to Ion-compatible adapters. Amplicon's end-repair and purification were performed according to the manufacturer's instructions. The samples were purified with Agencourt AMPure XP Kit for DNA purification on a DynaMag™-2 magnet magnetic rack (Thermo Fisher Scientific) following the procedure proposed by the manufacturer. The barcoding was done using Ion Xpress™ Barcode Adapters (Thermo Fisher Scientific). The same Ion Xpress™ P1 Adapter was ligated to the amplicons obtained from all the meat samples whereas a unique Ion Xpress™ Barcode Adapter for each sample was used. The samples were then purified with Agencourt AMPure XP Kit for DNA purification on a DynaMag™-2 magnet magnetic rack following the procedure proposed by the manufacturer. Libraries were amplified on an Applied Biosystems Veriti™ 96 well Thermal Cycler (Thermo Fisher Scientific) according to the manufacturer's instructions and quantified by a using the Qubit dsDNA HS Assay Kit to determine the molar concentration of each barcoded library, and then diluted as proposed by the Ion PGM™ Hi-Q™ sequencing Kit (Thermo Fisher Scientific). Emulsion

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PCR was performed following the manufacturer's protocol for the Ion OneTouch 2 Instrument (Life Technologies) to produce the required Ion Sphere Particles (ISPs). For sequencing chip, a pool of 30 µl containing equal amounts of each sample was prepared. The libraries were sequenced on the Ion Torrent PGM platform with an Ion PGM™ Hi-Q Sequencing 200 Kit. A sample was deemed to have a successful sequencing run if at least 50,000 reads with a quality score of Q20 (one misaligned base per 100 bases) were obtained, a minimum coverage of 500x was required.

Data analysis

The Ion Torrent PGM reads were compared to the GenBank nucleotide database. Ion Torrent PGM reads were collected by the Ion Torrent Suite software, which also sorted the data according to the barcodes. The software also scores the quality of the reads by assignment of Q20 scores according to the Ion Torrent's quality scoring computation. The percentage of genus identity and species identity value required at 97% and 99%, respectively.

FINDING AND DISCUSSION

To verify the usable of ribosomal RNA (12S and 16S rRNA) mitochondrial DNA gene, DNA of four meat species (cattle, chicken, fish, and pig) were used as templates for PCR amplification using adopted primer sets. Three adopted primer pairs (12S-Ki, 16S-KH, and 16S-Ki) were selected from the literature to amplify mtDNA regions (12S and 16S) containing species-specific information. All primer sets could be amplified DNA fragments of the four species according to their size expectation (Table 2). For 12S-Ki, agarose gel electrophoresis showed that three species (cattle, chicken, and pig) could generate PCR amplicons except one (fish). The PCR amplicons of 215-230 bp were amplified (Figure 1). For 16S-KH, agarose gel electrophoresis showed that all species (cattle, chicken, fish, and pig) could generate PCR amplicons. The PCR amplicons of 112 -130 bp were amplified (Figure 2). For 16S-Ki, agarose gel electrophoresis showed that two species (cattle and pig) could generate PCR amplicons except other two species (chicken and fish). The PCR amplicons of 243-253 bp were amplified (Figure 3). Unfortunately, the results showed that 12S-Ki and 16S-Ki universal primer could not be used to amplify in fish and chicken species. This occurs may due to the several mismatches between primer sequences and the annealed target regions of these species.

Table 2. Detection of meat species from 4 animals' DNA by conventional PCR.

Universal Primer	Cattle			Chicken			Fish			Pig		
	1	2	3	1	2	3	1	2	3	1	2	3
12S-Ki	+	+	+	+	+	+	-	-	-	+	+	+
16S-KH	+	+	+	+	+	+	+	+	+	+	+	+
16S-Ki	+	+	+	-	-	-	-	-	-	+	+	+

(+) amplification, (-) no amplification

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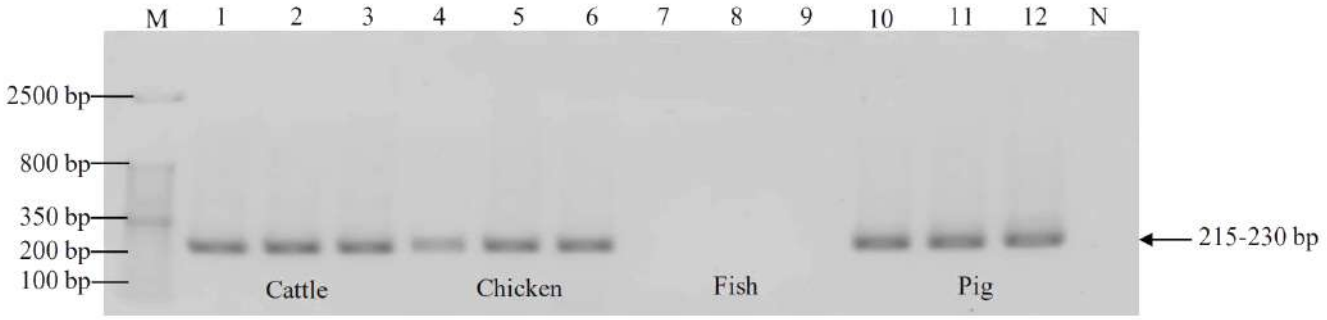

Figure 1. The image of PCR products using 12S-Ki universal primer from agarose gel electrophoresis. Lane M, 50bp DNA marker; Lane 1-3, cattle; Lane 4-6, chicken; Lane 7-9, Fish; Lane 10-12, pig; and Lane N, negative control.

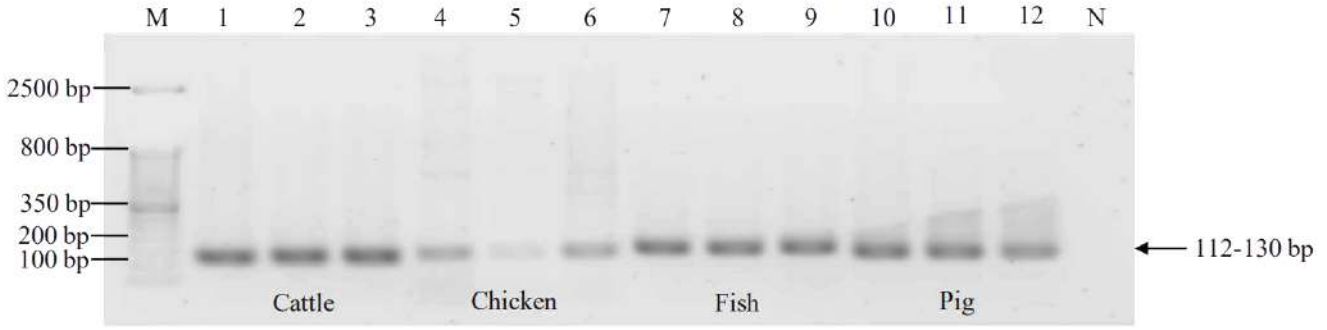

Figure 2. The image of PCR products using 16S-KH universal primer from agarose gel electrophoresis. Lane M, 50bp DNA marker; Lane 1-3, cattle; Lane 4-6, chicken; Lane 7-9, Fish; Lane 10-12, pig; and Lane N, negative control.

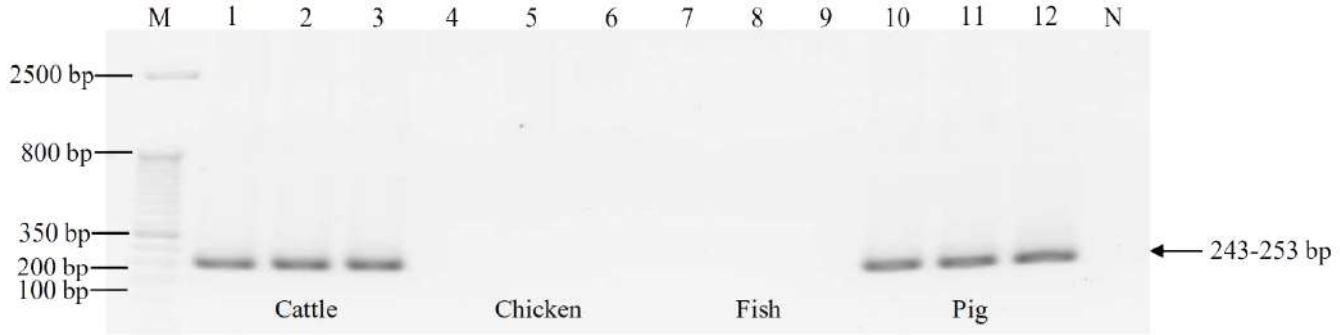

Figure 3. The image of PCR products using 16S-Ki universal primer from agarose gel electrophoresis. Lane M, 50bp DNA marker; Lane 1-3, cattle; Lane 4-6, chicken; Lane 7-9, Fish; Lane 10-12, pig; and Lane N, negative control.

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To verify the usable of NGS, the libraries of the amplified PCR amplicons were constructed and sequenced. The amplicons obtained from the conventional PCR step were prepared in library 1 to 12 and sequenced with the Ion Torrent PGM. The number of mapped reads to the corresponding reference mtDNA regions (12S-Ki, 16S-KH and 16S-Ki), without (Total reads) and with preliminary filtering steps (Valid reads) is shown in Table 3. For 12S-Ki, the amplicons with library 1 to 4 were sequenced. The results of BLAST showed 2,514 reads (37.91%) in cattle, 1,739 reads (26.23%) in chicken, 0 reads (0.00%) in fish, and 2,378 reads (35.86%) in pig. For 16S-KH, the amplicons with library 5 to 8 were sequenced. The results of BLAST showed 6,285 reads (22.56%) in cattle, 3,959 reads (14.21%) in chicken, 9,207 reads (33.05%) in fish, and 8,409 reads (30.18%) in pig. For 16S-Ki, the amplicons with library 9 to 12 were sequenced. The results of BLAST showed 10,848 reads (76.75%) in cattle, 0 reads (0.00%) in chicken, 0 reads (0.00%) in fish, and 3,286 reads (23.25%) in pig (Table 3 and Figure 4). These results were similar to the previous studies of Liu et al. (2021) and Bertolini et al. (2015). The NGS coupled with rRNA mitochondrial DNA gene had good detection effect on mammalian species, but it had poor detection effect on poulty species. Interestingly, amplicons amplified by 12S-Ki (fish) and 16SKi (chicken and fish) could not be mapped to the mtDNA database. This demonstrated that NGS coupled with rRNA mitochondrial DNA gene had a high potential to identify meat species since the amplicons could not be amplified by conventional PCR. In this study, 16S-KH showed better detection effect in all species comparing with others. This demonstrated that 16S-KH was reliable and usable for meat species identification. Therefore, it was chosen to further use in precision (reproducibility) of meat species identification using NGS.

Table 3. NGS sequencing results by 12S-Ki, 16S-KH, and 16S-Ki primer.

Primer name	Species	Base	Q20	Total Read	Valid read	mapped read
12S-Ki	Cattle	1,370,239	1,224,901	7,671	6,636	2,514
	Chicken	943,856	851,071	5,235	4,450	1,739
	Fish	2,435,841	2,188,886	13,540	11,714	0
	Pig	1,519,488	1,327,858	8,260	7,480	2,378
16S-KH	Cattle	1,089,953	1,004,640	10,961	9,267	6,285
	Chicken	840,607	766,884	7,548	6,991	3,959
	Fish	1,863,969	1,708,795	15,725	14,281	9,207
	Pig	1,614,247	1,442,022	16,106	13387	8,409
16S-Ki	Cattle	5,294,863	4,888,586	24,623	22,539	10,848
	Chicken	2,460,738	2,262,428	11,605	10,373	0
	Fish	2,202,543	2,035,535	10,305	9,280	0
	Pig	1,895,529	1,752,051	8,956	8,042	3,286

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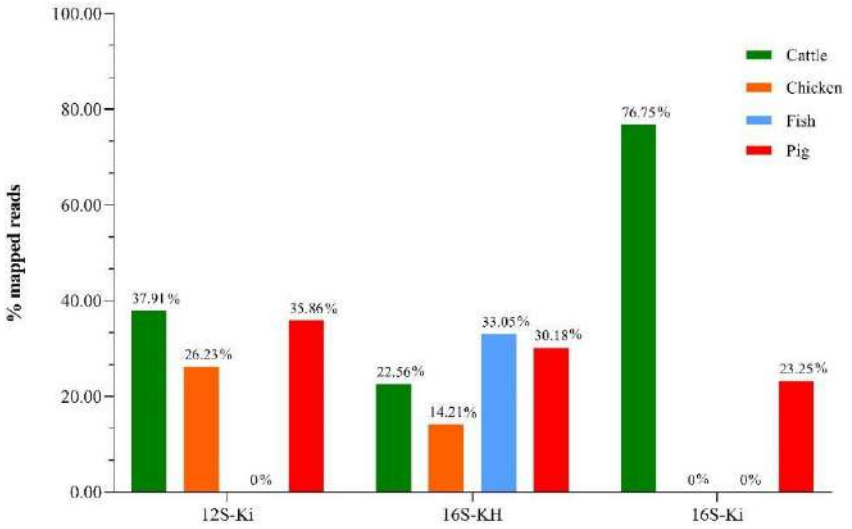

Figture 4. NGS sequencing results of 4 meat species (cattle, chicken, fish, and pig) amplified by 3 pairs of adopted primer.

For precision evaluation, the 16S-KH primer was further used to amplify the targeted PCR amplicons from the four meat species. The amplicons obtained from the conventional PCR step were prepared in library 1 to 4 and then sequenced with the Ion Torrent PGM. The experimental precision was directly investigated comparing the results obtained from library 1 to 4 that derives from DNA of four meat species which separately amplified for 3 different runs (Table 4). The number of mapped reads to the corresponding reference mtDNA regions (16S-KH), without (Total reads) and with preliminary filtering steps (Valid reads) is shown in Table 4. For 1st run, the amplicons with library 1 to 4 were sequenced. The results of BLAST showed 7,853 reads (24.93%) in cattle, 4,425 reads (14.04%) in chicken, 9,779 reads (31.04%) in fish, and 9,447 reads (29.00%) in pig. For 2nd run, the amplicons with library 1 to 4 were sequenced. The results of BLAST showed 7,573 reads (25.70%) in cattle, 4,461 reads (15.14%) in chicken, 9,422 reads (31.98%) in fish, and 8,007 reads (27.18%) in pig. For 3rd run, the amplicons with library 1 to 4 were sequenced. The results of BLAST showed 6,285 reads (22.56%) in cattle, 3,959 reads (14.21%) in chicken, 9,207 reads (33.05%) in fish, and 8,409 reads (30.18%) in pig. The percentage of mapped reads ranged from 14.04% to 31.04%, 15.14% to 31.98%, and 14.21% to 33.05% (1st, 2nd, and 3rd run, respectively) (Figure 5). As expected, the number and proportion of mapped reads of each species per run were similar. Besides, the number and proportion of mapped reads between three different runs were also concordant (Figure 5). This demonstrated that NGS coupled with rRNA mtDNA gene could be reliably implemented as a routine testing of meat inspection and authentication.

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Table 4. NGS sequencing results by 16S-KH primer.

Run no.	Species	Base	Q20	Total Read	Valid reads	Mapped reads
1st	Cattle	1,035,782	1,026,913	22,980	21,991	7,853
	Chicken	981,549	887,534	10,844	9,973	4,425
	Fish	1,588,948	1,500,638	20,541	18,368	9,779
	Pig	1,456,547	1,382,846	19,335	18,471	9,447
2nd	Cattle	1,163,981	1,017,913	20,367	19,992	7,573
	Chicken	1,230,290	1,148,544	11,744	10,584	4,461
	Fish	1,617,893	1,438,847	24,951	24,011	9,422
	Pig	1,241,337	1,036,884	28,561	27,877	8,007
3rd	Cattle	1,089,953	1,004,640	10,961	9,267	6,285
	Chicken	840,607	766,884	7,548	6,991	3,959
	Fish	1,863,969	1,708,795	15,725	14,281	9,207
	Pig	1,614,247	1,442,022	16,106	13,387	8,409

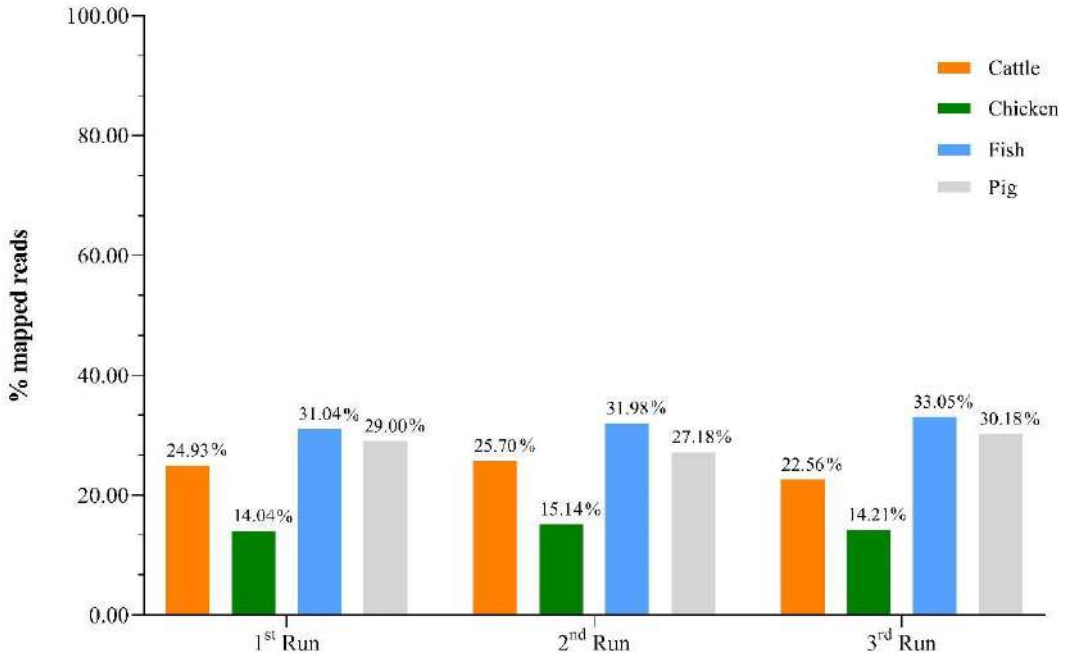

Figure 5. NGS sequencing results of 4 meat species (cattle, chicken, fish, and pig) amplified by 16S-KH primer.

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CONCLUSION AND FURTHER RESEARCH

In this study, we tested the possibility to use the NGS technology coupled with ribosomal RNA mitochondrial gene to identify meat species including cattle, chicken, fish, and pig. The results showed that NGS coupled with 16S-KH primer had a positive detection effect on all meat species. However, the values of mapped reads were still low. Hence, PCR quantity and quality protocol should be further optimized. In addition, the good laboratory practices should be implemented. DNA extraction, PCR, and NGS sequencing should be performed in different rooms to avoid accidental contamination between samples. Moreover, other halal and haram species should be further examined for comprehensive test of meat inspection and authentication. This innovative technique could be applied to employ as a monitoring tool for identifying food fraud and undeclared meat species. It may become a routine method for species identification in meat products and will build confidence in the safety of halal food products for consumers.

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