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ORIGINAL ARTICLE

A simple and sensitive NGS-based method for pork detection in complex food samples

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Abstract Food adulteration is a serious concern faced by the importers of various food products across the globe. In this study, a simple, sensitive and robust method for detecting pork in processed/complex food samples using next-generation DNA sequencing (NGS) technology is described. The experimentation involves a generalized library preparation kit for performing shotgun sequencing of the genomic DNA irrespective of its intactness. The method was applied on different complex food samples containing pork along with other species (up to twelve) as well as without pork to test the specificity of the method. The DNA sequences were mapped with the online NCBI nucleotide database for their identification followed by a calculation of the relative abundance of the reads. The adulteration of pork was correctly identified in the analyzed samples. Although the relative abundance of pork DNA reads could not make a precise quantitative

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relevance with the contributed amount of the tissue sample, yet this method has the potential to determine extremely low as well as high contents of adulterating/contaminating species in complex food products.

1. Introduction

Due to the high demand and mass consumption of meat products all over the world, meat production and export has become a growing business worldwide which has unfortunately, lead to adulteration and food fraud. After the European horse meat scandal in 2013 (Cottenet et al.,2020), food adulteration and species substitution irregularities have gotten full attention. Various survey and research studies have pointed out that the mislabeling of the meat was not only faced in Europe but also in other countries such as Canada (Naaum et al., 2018), South Africa (Cawthorn et al., 2013), United States (Kane & Hellberg, 2016; Quinto et al., 2016) and Malaysia (Chuah et al., 2016).

Pork is considered the cheapest meat and widely used as a substitute for expensive meat despite health-related concerns and prohibition in some religions (Alves et al., 2020; Rohman & Che Man, 2012; Djurković-Djaković et al., 2013; Franssen et al., 2017; Gajadhar et al., 2018; Gamble, 1997; Meester et al., 2019; Zhang et al., 2019). Therefore, the identification of pork in food products is an important issue to consumers and food authorities (Ayuso et al., 1999; Mamikoglu, 2005; Schäfer et al., 2001). Several analytical methods are available for confirming and verifying the authenticity of the meat products and to make sure that the detected animal species is the one that is declared (Staats et al., 2016). DNA-based testing has become the most effective approach in certifying both the animal origin and quality of raw materials, and to detect adulterations occurring in the industrial food chain. Due to its specificity, PCR-based DNA analysis is the most frequent tool that is used to test the presence of pork DNA in products (Raharjo & Rohman, 2016; Rahmawati et al., 2016).

Next-generation DNA sequencing (NGS) is an advanced approach that provides a massively parallel and extremely high-throughput analysis of multiple samples. It has enabled the sequencing of millions of DNA fragments simultaneously. The cost per NGS experiment has also reduced significantly these days (Staats et al., 2016). NGS is becoming more popular for testing food authenticity (Haynes et al., 2019) and recent studies have shown its applicability for seafood (Giusti et al., 2017), spices and herbs (Barbosa et al., 2019), and meat species identification (Ribani et al., 2018; Xing et al., 2019). It provides new opportunities for the identification of species composition in a complex mixture (Ribani et al., 2018).

In previous studies, the detection of pork has been reported by different DNA-based methods including real-time PCR, isothermal amplification, digital PCR, biosensors, DNA metabarcoding and NGS etc. (Staats et al., 2016; Wu et al., 2020; Xing et al., 2019). However, these reported methodologies either cannot be efficiently applied to complex food samples (where the target gene/locus is not intact) or require higher computational resources. Various approaches employing the DNA sequencing of targeted regions such as cytochrome *c* oxidase submit 1 (*COX1*) and 16S rRNA gene have been adopted to identify meat species but these face technical challenges because these strategies rely on amplification of the target regions followed by the sequencing to identify the source of meat and poultry products (Handy et al., 2011; Sarri et al., 2014; Xing et al., 2019). Compared with the enrichment of targeted regions and detection methods, the whole genome shotgun sequencing-based methods (Cottenet et al., 2020; Haiminen et al., 2019) are more sensitive. However, these may be more expensive because they require the building of local databases of potential targeted species. Further, the organisms without a reference sequence in the custom database could not be traced by using these approaches.

Herein, we devised a simple protocol using a non-customized DNA library prep kit that utilizes the genomic DNA (intact or degraded) for shotgun sequencing and can be used for identification of pork in complex food samples containing meat as well as other tissue/body part containing the DNA. Our reported method can be applied to technically challenging food samples where targeted region-based PCR strategy could not work. We have explained the NGS application for the detection of pork DNA in complex and technically difficult food mixture. Moreover, we demonstrate that a small amount of sequencing data can be utilized to make the protocol cost-effective yet maintaining its specificity.

2. Material and methods

2.1. Samples preparation

For determining the pork adulteration in the foodstuff using the next-generation DNA sequencing (NGS) technology, five complex admixed samples were prepared by mixing a variety of meat sources used as food in different countries/ethnicities of the world. Details of admixing constituents in each sample are presented in Table 1. For the admixed samples preparation, about 2.0 g of each of the components was mixed and homogenized for constituting a group. Four admixed samples were pork positive containing pork as well as varying tissue parts of different animal species, and one admixed sample was pork negative, which was included as a negative control to assess the specificity of the assay.

2.2. DNA extraction, quantification and quality assessment

The genomic DNA (gDNA) was extracted from the samples using QIAGEN DNeasy Mericon Food Kit (QIAGEN, Germantown, MD, US). Approximately, 2.0 g of each admixed sample was homogenized in liquid nitrogen using mortar and pestle, and the gDNA was extracted following the manufacturer's protocol. The quantity of the isolated gDNA was determined by the Qubit High-Sensitivity dsDNA assay kit (Thermo Fischer Scientific, MA, US). The quality of the isolated gDNA was assessed with 1% agarose gel electrophoresis. The purity of the gDNA was assessed by determining A260/A280 and A260/A230 ratio using NanoDrop™ 1000 spectrophotometer (Thermo Fischer Scientific, MA, US).

2.3. Library preparation for DNA sequencing

DNA libraries were prepared using Illumina Nextera DNA library prep kit (Illumina, San Diego, CA, US) following the manufacture's protocol. Briefly, 50 ng of gDNA of each sample was subjected to tagmentaion followed by the addition of DNA adapters in a single enzymatic reaction. The tagmented DNA was purified using Agencourt AMPure XP beads (Beckman Coulter, CA, US). To achieving samples multiplexing in a single sequencing run, unique dual index adapters (i5 and i7)

Table 1 Sources of foodstuff in five samples used for source identification.

Sample 1 (pork positive)	Sample 2 (pork negative)	Sample 3 (pork positive)	Sample 4 (pork positive)	Sample 5 (pork positive)
Australian parrot feather	Chicken meat (uncooked)	Australian parrot feather	Camel meat (uncooked)	Camel meat (uncooked)
Chicken meat (cooked)	Cow meat (uncooked)	Chicken meat (cooked)	Cat blood serum	Cat blood serum
Fish meat (uncooked)	Finch feather	Chicken meat (uncooked)	Chicken meat (cooked)	Chicken meat (cooked)
Ostrich egg	Ostrich egg	Cow meat (uncooked)	Chicken meat (uncooked)	Chicken (uncooked)
Pork meat (uncooked)	Shrimp meat (uncooked)	Finch feather	Cow meat (uncooked)	Cow meat (uncooked)
Quail meat (uncooked)	Veal meat (uncooked)	Fish meat (uncooked)	Fish muscle meat (uncooked)	Fish muscle meat (uncooked)
		Ostrich egg (raw)	Pork (uncooked)	Duck egg
		Pork meat (uncooked)	Quail meat (uncooked)	Ostrich egg
		Quail meat (uncooked)	Shell fish meat (uncooked)	Shell fish meat (uncooked)
		Shrimp meat (uncooked)	Shrimp meat (uncooked)	Quail meat (uncooked)
		Veal meat (uncooked)	Veal meat (uncooked)	Pork (uncooked)
				Shrimp meat (uncooked)
				Veal meat (uncooked)

were added to the tagmented DNA of each sample in a limited number of cycles of PCR reaction (6 cycles) using the Illumina Nextera index adapters (Illumina, San Diego, CA, US). The indexed DNA was again purified using Agencourt AMPure XP beads yielding the DNA libraries for sequencing with Illumina sequencers. The quality of DNA libraries was assessed with real-time PCR using the QIAGEN Quant Library assay kit (QIAGEN, Germantown, MD, US), and the number of DNA libraries was assessed with the Qubit High-Sensitivity dsDNA assay kit. For performing DNA sequencing, equimolar libraries were mixed in a single microcentrifuge tube, followed by DNA denaturation with 0.2 N NaOH, and dilution to 10 pM with ice-chilled HT1 hybridization buffer. Single-end sequencing (100 bp) was performed with Illumina MiSeq (Illumina, San Diego, CA, US) using the MiSeq Reagent cartridge v2 kit (MS-102–2002).

2.4. NGS data analysis

DNA sequences were de-multiplexed and retrieved in 'fastq' format from the MiSeq. For analysis of the DNA short reads, we developed a simplified analysis pipeline in the Linux operating system environment. This pipeline involves the matching of DNA reads with an online nucleotide database of thousands of organisms. The quality of short reads was assessed using the FastQC tool (Andrews, 2010). Few nucleotide bases from the 3′-prime of the reads were trimmed using the Trimmomatic tool (Bolger et al., 2014) to improve the average quality score of reads and subsequent downstream processes. To determine source organisms in foodstuff, we used a standalone blastn tool (BLAST+) of the National Center for Biotechnology Information (NCBI) (Camacho et al., 2009). The blast analysis of short reads was performed remotely against the NCBI non-redundant nucleotide database. To minimize mismatching and matching with lower identity, only best hit was allowed with a minimum percent identity of 99%, query coverage of 99%, and maximum matching with 5 organisms. The relative abundance of contributing species/organisms was determined from the blast output. The relative abundance represents the fraction of DNA reads matching with specific species to the total DNA reads of a sample. The code used for the analysis is presented in Supplementary File 1.

3. Results

3.1. Complex food samples and working strategy

The present study was designed to determine the adulteration of pork in complex foodstuff by using next-generation DNA sequencing with minimal experimental work and bioinformatics analysis. In the present study, the samples contained muscle meat, including cooked and raw meat, blood, feather tip and egg of different species to make the admixed samples quite complex and evaluate the capability of NGS to correctly detect and identify pork DNA simultaneously. The food mixtures were prepared to contain adulterant species spiked as low as $<1\%$ (w/w) (e.g., feather tips), which is even less than the pragmatic threshold defined by the European Union recommendation (European Commission, 2013). The sample preparation (library construction) for DNA sequencing was carried out in less than a day. In the sequencing run, 750 Mb (mega bases) data was generated, where the five samples contained 403,599, 551,877, 515,299, 335,921, and 770,946 pass-filter DNA reads, respectively. The de-multiplexing of the samples and generation of FASTQ files was achieved on the instrument using the built-in MiSeq control software. For pork identification, the absolute abundance of the blast hits (reads) for each organism was determined and the percentage was calculated for the proportion of pork contribution (Supplementary Tables 1-5). A schematic diagram of the workflow is presented in Fig. 1.

3.2. Samples analysis

All the tested samples led to correct detection and identification of pork. The samples, which contained mixtures of different tissues of more than five species, also lead to the correct

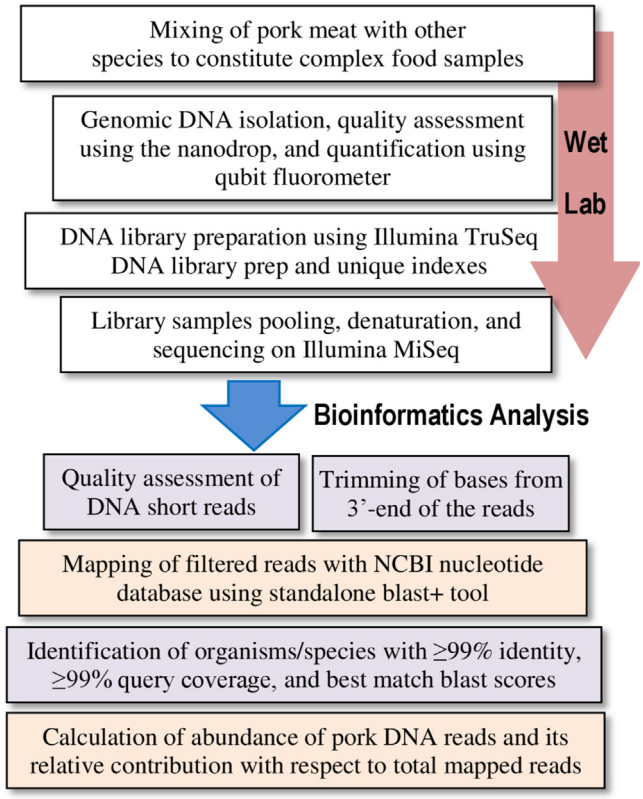

Fig. 1 Workflow for the samples preparation and DNA sequencing followed by bioinformatics pipeline for detecting pork in the complex food samples.

identification of pork. The subjects matched with the Expect Value (E-value) of below 1.0×10^{-4} were retained. The E-value describes the number of hits one can expect to see by chance when searching a database of a particular size. The lowest E-value represents the most accurate matching during the blast analysis. Since the sequencing results were found in the form of a large number of short reads, it was deemed convenient to quantitatively correlate the number of correctly mapped sequencing reads and the amount of sample spiked in the mixture. The relative abundance of reads was determined by calculating the fraction of DNA reads mapped with the pig genome to the total number of reads in a given sample. However, the relative abundance of pork DNA reads in the admixed samples could not be correlated quantitatively with the amounts of spiked constituents in the sample preparation (Fig. 2). We calculated deviance of the calculated relative abundance of pork DNA reads in total reads from the proportion of meat being admixed in the sample preparation. In Sample 1 and Sample 5, the relative abundance of pork DNA reads was 2.19% and 2.24% less than the percent amount of pork added in the admixed sample, respectively. In Sample 3 and Sample 4, the relative abundance of pork DNA reads was 4.4% and 1.15% greater than the percent amount of pork added in the admixed sample, respectively. This disagreement may be due to the technical reasons that different types of tissues, due to their diverse biological natures, give different yields in DNA isolation. For example, DNA yield from a given quantity of muscle meat can be considerably higher than the comparable quantity of feather, bones, fish gills etc. (Ballin et al., 2009). Also, the processing of food can impact the integrity of DNA. Temperature, pressure, and pH are the most frequent industrial parameters which can affect DNA quality in food items (Hird et al., 2006). Nevertheless, the spiked pork was successfully detected qualitatively in the analysis.

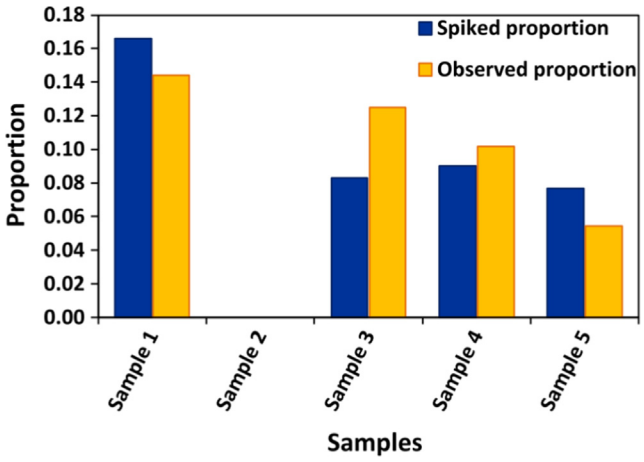

Fig. 2 Comparison between the amounts of pork being added in different samples and observed relative abundance of pork reads matched with pig genome sequences. The Spiked proportion represents the amount of biological tissue added during the preparation of admixed samples, whereas, Observed proportion represents the relative abundance of pork DNA sequencing reads in each sample. Sample 2 was included as a negative control, in which pork was not added during the sample preparation.

4. Discussion

The present study was designed to determine the pork adulteration in technically challenging foodstuff using minimal experimental work and bioinformatics analysis. Next-generation DNA sequencing using the Illumina MiSeq was employed for the current study. This approach is simple and can be applied to complex samples including cooked/processed tissue ingredients. With optimized consumables and equipment in a laboratory, the sample preparation would take one day, followed by overnight sequencing, and data analysis on the next day. For massively parallel sequencing, Illumina MiSeq was utilized in this study, but we presume that other comparable instruments for DNA sequencing such as Ion PGM or Oxford nanopore MinIon can also be employed for this purpose. Furthermore, contrary to the classical approach of qPCR, which is based on amplification and detection of a limited number of target regions, this NGS approach can identify pork from complex mixtures, along with adulterating species even when the sample is in processed/cooked form. Hence, it expands the scope of accurate molecular identification of pork in a single experiment within a limited time.

In recent years, several approaches using DNA sequencing have been presented to identify meat species but these face technical challenges e.g., the method presented by Handy et al. (2011) targets gene sequence of cytochrome *c* oxidase subunit I (*COXI*) which could be difficult to amplify in a polymerase chain reaction in degraded or highly processed samples. Sarri et al. (2014) proposed sequencing of 16S rRNA gene for species identification, which could also be difficult

to amplify in degraded samples; and very closely related species might not be discriminated using the proposed 16S rRNA gene sequence. Recently, the method of 'DNA metabarcoding' of 16S rRNA gene proposed by Xing et al. (2019) to identify the source of meat and poultry products could only be applicable on fresh samples. Contrary to these targeted region sequencing method, a commercially available whole genome shotgun sequencing-based customized kit 'All Species ID MEAT DNA Analyser kit' is available to identify the source of meat in food samples (Cottenet et al., 2020), yet it can be utilized with the designated instrument only. In the present study, we have come up with a simple protocol using a generalized library preparation kit. The method relies on the genomic DNA, irrespective of its intactness, for shotgun sequencing. Moreover, the sensitivity of protocol was achieved with quite low volume of data (~0.5 million DNA reads per sample) to make the protocol cost-effective. This approach also allows multiplexing of a large number of samples in a sequencing run to enhance the per batch samples throughput. Also, the method is not limited to the detection of pork, it can be employed for detection of any adulterant species after optimization of the 'blastn' search parameters, and increasing the sequence length of DNA reads to improve species resolution. Comparison of the proposed method in this study with the previously reported methods has been presented concisely in the Table 2.

Beyond the cost, the method can be used for the identification of adulterant pork in complex and technically challenging food samples containing different parts of organisms including muscle meat, feather tip and eggs, where the traditional PCR may fail to detect the adulterant pork. A very similar approach of shotgun sequencing was adopted previously by Haiminen et al. (2019) but they built a local database of >6000 plants and animal species that are potentially used as food. The building of local databases requires high computation resources and may be difficult for laboratories with limited computation infrastructure. Further, the organisms whose reference sequence was not included in the custom database could not

Table 2 Comparison of the proposed method in the current study with the previously reported methods of pork detection in foodstuff.

Previous Study	Method used	Challenges/Disadvantages	Advantages
Handy et al., 2011	Sequence of cytochrome *c* oxidase subunit I (*COXI*) gene	• It is difficult to amplify the target gene through PCR in degraded or highly processed samples. • Processing of a large number of samples could be laborious.	• Cost-effective. • The analysis of test results is simple
Sarri et al., 2014	Sequencing of 16S rRNA gene for specie identification	• It is difficult to amplify the target gene through PCR in degraded or highly processed samples. • Processing of a large number of samples could be laborious.	• Cost-effective. • The analysis of test results is simple.
Xing et al., 2019	DNA metabarcoding of 16S rRNA gene	• This method can be applied to fresh samples only.	• Cost-effective. • The kit used is a generalized one and can be used with multiple NGS platforms.
Cottenet et al., 2020	Shot-gun sequencing of multiple genes	• This method is based on a customized kit 'All Species ID MEAT DNA Analyser kit'. • Only the designated instrument can be used for this method.	• Technically challenging samples can be analyzed by this method • The sensitivity is higher than the PCR-based method. • This method can handle fresh, stored, processed, and degraded samples.
Haiminen et al., 2019	Shot-gun sequencing of multiple genes	• This approach requires building a local database of >6000 plants and animal species. The building of local databases requires high computation resources and would be costly. • Species not included in the database cannot be detected	• Technically challenging samples can be analyzed. • This method can handle fresh, stored, processed, and degraded samples • The kit used is a generalized one and can be used with multiple NGS platforms
Our proposed method	Shot-gun sequencing of multiple genes	• Continuous availability of the internet during 'blast' analysis. • It requires the manual assessment of data to overcome multiple species identified with the same reads and quality scores due to conserved regions.	• Highly processed and technically challenging samples can be analyzed • This method can handle fresh, stored, processed, and degraded samples • The kit used is a generalized one, and can be used with multiple NGS platforms • Minimum computational resources are required

be traced by using this approach. Contrary to this, the approach described in the present study did not require building a local database of selected organisms making it inexpensive computationally.

5. Conclusion

This study presents a simple yet sensitive method using minimal experimental and bioinformatics analysis infrastructure for the detection of pork in food samples using the next-generation DNA sequencing technology. This method successfully identified the spiked pork qualitatively in admixed samples containing up to 12 different species. The availability of a stable internet connection for the blast search is the only limiting factor of this study. The quantitative accuracy of the method can be achieved by increasing the length of sequencing reads, may be sequencing of 150x2 bp could fulfill the purpose. Compared with other related studies involving NGS, our method is more cost-effective in terms of laboratory consumables and bioinformatics analysis infrastructure. Taken together, our approach can be applied for the detection of food adulteration with pork in laboratories with limited a bioinformatics setup.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103123.

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