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Original Research Article

Biomedical and Halal Aspects Counterfeiting of Meat on Processed Food in Bali and Nusa Tenggara Indonesia, 2021

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Abstract: Based on Law no. 18/2009 of the Republic of Indonesia concerning Animal Husbandry and Animal Health that food of animal origin must be safe, healthy, whole and halal. From one aspect, the content of pork and rat meat in processed food products is an act of counterfeiting that is detrimental to consumers and this is against the law because of allergy and halal issues. In practice, meat adulteration by means of mixing pork or rodent derivatives with beef or chicken had been frequently detected by using molecular technology in some market in Java, Indonesia. Unfortunately, very rare information has been available in Bali and Nusa Tenggara. The purpose of this study was to confirm the meat adulteration issues in the region, by analyzing the surveillance data provided by the Disease Investigation Center Denpasar Indonesia. A total of 181 various kinds of samples were collected during active and passive surveillance, although only 85 samples consisting of various animal products namely meatballs, sausages, burgers and fresh meat were analyzed and reported in this study. The method used to obtain the samples were food safety key indicators approach and tested to detect species-specific, cytochrome b (cyt b) coding genes of porcine and rodent. For this, genomic DNA of porcine and rodent were isolated and subjected to PCR amplification using specific set of primers for each animal species. The PCR test showed that a box of meat sample that was previously thought to be beef was PCR positive to contain porcine DNAs. However, all of the animal products tested were negative PCR, suggesting no evidence of pork and rodent meat ingredients in the processed foods tested. It was concluded that the molecular technique could specifically be used to confirm meat counterfeit. This finding confirmed there was a degree of security guarantee for meat adulterations in processed food but not in fresh beef which has medical aspect for allergic consequences and *halal fatwa*.

Keywords: Halal, PCR, Primers, Pork, Rodents.

INTRODUCTION

The advances in molecular biology, especially the use of polymerase chain reaction (PCR), had helped to detect various genetic materials at the RNA/DNA levels. Its use is no exception to detect adulteration meats in processed foods that leads to several fears for the Muslim community [1]. In Indonesia, the meat used to make processed foods such as meatballs, sausage and floss have originally come from beef. Nowadays, however, some other meats originated mainly from chicken, fish, pork, and rodent are also commonly used in some of the products [2]. This is a challenge for officials in charge who have obligation to verify the species of meat ingredients in the food that are not always easily identified. In the past, to detect the adulterated products had been based on wet chemistry to test the amount of a marker compound in tested materials and compared with the value(s) obtained from authentic material of the same type. This old method is considered less feasible, time-consuming, less practical, and relatively expensive. Fortunately, in recent decades, some molecular-based analytical methods can be applied to overcome the problems that offer fast and reliable results, for example, DNA-based methods [3]. The methods have widely been developed such as multiplex PCR assay [4], PCR-based fingerprinting [5],

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and also real-time PCR used by [6, 7]. The methods were mainly used to detect meat bone meal, meat source origin, and to quantify meat in DNA mixture complex. Furthermore, works done by [8] and [9] who identify DNAs origin of the source of meat from which species, by using species-specific primers will be very useful for future research. Recent work published the use of a specific primer to detect cytochrome b (Cyt b) coding gen of porcine that produced 149 bp of amplified DNA fragments [10]. Detection of the Cyt b gene was a valuable gene used for molecular phylogenetic relationships of retain species [11]. Therefore, the aim of this study was to use conventional PCR technology to detect specific porcine and rodent DNAs in suspected samples obtained from several different places in Bali and Nusa Tenggara of Indonesia.

MATERIAL AND METHODS

The location of sampling was determined using the purposive method, the location was predetermined. Type of targeted location including markets and retailers (meat, processed meat), and meat importing companies was done randomly. During two consecutive years of surveillance 2018 to 2019, a total of 180 samples were collected, although only 84 of them originated from varied-animal products and were specially analyzed using molecular methods (Table 1).

Table-1: Details origin and type of samples obtained during the surveillance.

Years	Provinces	Sampling places	Type of samples	Total
2018	Bali	Retail/market	Meatball	34
	West Nusa Tenggara	Retail/market	Meatball	10
	Eastern Nusa Tenggara	Retail/market	Meatball	10
2019	Bali	Food distributor	Meatball	12
		Meat storage	Sausages	8
	Eastern Nusa Tenggara	Meat distributor	Fresh beef	1*
		Cold storage	Meatball	4
		Cold storage	Sausages	6
Total				85

Note *: 1 box sample of fresh beef passively sent to Disease Investigation Center Denpasar.

DNA was extracted from meatball or sausages presented on Table 1 using protocol provided by the Qiagen DNeasy blood and Tissue Handbook 07/2020 (Qiagen USA), with slightly modification as follows. Firstly, water bath/heat block was prepared at 55 ^{0}C and 70 ^{0}C then about 25 mg of samples was put into microtubes. Subsequently 180 µl of lysis buffer (L6) and 20 µl of proteinase K were added into the microtubes, and incubated at 55^{0}C for 30 minutes. Before the tubes were further incubated at room temperature for 2 minutes, a total of 20 µl of RNase was added into the tubes. This step was continued by centrifugation of the microtubes at 13,000 rpm for 5 minutes. The supernatant was transferred to a new tube and 10 µl of 10% SDS was added and vortexed. After this step, 200 µl of binding Buffer (L3) was added to the tube and vortexed and incubated at 70^{0}C for 10 minutes and followed by the addition of 200 µl of 90-100% ethanol into the tube. Furthermore, the liquid in the tube was transferred to the spin column and centrifuged at 12,000 rpm for 30 seconds. The liquid was removed and W4 was added again and then centrifuged at 12,000 rpm for 30 seconds. The liquid was discarded, 500 µl of wash buffer (W5) was added to the tube then at 12,000 rpm for 30 seconds and repeated once again. The liquid was discarded and centrifuged at 12,000 rpm for 30 seconds. Place it in a collection tube and add 100 µl of elution buffer (E1) and incubate at room temperature for 1 minute and finally centrifuged at 13,000 rpm for 2 minutes and the isolated DNA samples were stored at 4^{0}C before amplified by PCR.

PCR assays were performed using the methods by [12] and [10]. Set of primers sequence used were Forward primer 5' CTACGGGTCTGTTCCGTTGG 3' 149 bp Reverse Primer 5' ATGAAACATTGGAGTAGTCCTACTATTTACC 3. The formula of master mix used as provided by Winarsih et al 2018 with slight modification that were consisted of 25 µL Go Tag (*Taq Polymerase*, MgCl2, and DNTPs), 10 µL cytb forward primers, 10 µL reverse primer and 13 µL of nuclease free water. Total volume per PCR reaction was 50 µL including 40 µL of master mix and 10 µL of 50 ng DNA sample. Amplification was performed with a Thermal Cycler (Mastercycler Gradient, Eppendorf) according to the following PCR step-cycle program: pre-denaturation of 98°C for 2 min to completely denature the DNA template, continued by 50 cycles, consisted of an initial denaturation step for 30 second at 95°C, 30 second annealing at 60 °C, 40 second polymerization at 72 °C ending with a final 3 min extension at 72 °C. Subsequently, 10 µL of each amplified product and 10 µL of DNA control was subjected into 1% of agarose gel (Promega, Madison, USA) and electrophoresed at 100 volts, 400 ampere for 75 minutes in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A-100 bp DNA ladder (Promega, Madison, USA) was used as size reference. The gel photo was taken using the Syngene gel documentation system.

To detect adulteration of rodent meats, DNA extraction was carried out using Purelink Viral RNA/DNA mini kit. Firstly, 20% (w/v) suspension of samples was made by cutting it into small pieces, weighed as much as 0.2 grams, crushed and diluted by 1 ml of 1% PBS. Subsequently, a total of 200 ul of the sample suspension was put into a 1.5 ml tube and mixed with 200 ul of lysis buffer and 24 ul of Proteinase K. The suspension was vortexed and incubated in a water bath at 56^0C for 15 minutes. To the mixture above, 200 ul of ethanol was added and incubated at room temperature for 5 minutes. The suspension was transfer into a spin column and centrifuged at 10,000 rpm for 1 minute. The liquid in the collection tube was discarded. Next, 500 ul of washing buffer was poured into spin column connected with a new filtrate collection tube and centrifuged at 10,000 rpm for 1 minute. The filtrate collection tube was replaced and centrifuged at 10,000 rpm for 1 minute, followed by the addition of 50 ul of nuclease free water (NFW) into the spin column connected with new and sterile 1.5 ml recovery tube. Finally, the column was again centrifuged at 12,000 rpm for 1 minute, and extracted DNAs in the recovery tube can be directly (immediately) or can be stored in a freezer at a temperature of -20^0C before further amplified by PCR. To analyze rodent DNAs, PCR assay was based on method of [13]. Primer sequence used to detect the Cyt b gene were forward primers: 5'CAT GTG GGA CGA GGA CTA TAC TATG-3' and reverse primers: 5'GTA GTC CCA ATG TAA GGG ATA GCTG-3'. The total volume of the reaction mix in the PCR tube was 25 µl consisting of 21 µl Platinum Blue Supermix, 1 µl forward and reverse primers with a concentration of 20 pmol and 2 µl of template DNA. The thermocycler was performed for 35 cycles. with the following program: pre denaturation at 95^0C for 5 minutes, followed with denaturation at 95^0C for 30 seconds, annealing at 60^0C for 45 seconds and extension at 72^0C for 1 minute. To visualized the amplified DNAs, 10 µL of each amplified product and 10 µL of DNA control was subjected into 1.5% of agarose gel (Promega, Madison, USA) and electrophoresed at 100 volts, for 1 hr in 1x TBE buffer, pH 8.0 and stained by sybrsafe. A-100 bp DNA ladder (Promega, Madison, USA) was used as size reference. PCR amplification products gel was visualized over UV transilluminator to take photos.

RESULT

Of the total 85 samples tested that consisted of 84 processed product (meatballs, sausages) and a box of samples suspected to be pork were successfully analyzed using PCR. Using species-specific set of primers for porcine and rodent, all of the 84 process products were negative, not to contain any target Cyt b genes of the two species (Table 2). Agarose gel electrophoresis of the negative PCR-products (not shown). However, the suspected pork sample that was administratively sent from Java by meat distributor was confirmed PCR positive to contain porcine gene. The positive PCR product showed a specific amplified porcine DNA of about 149 bp and high range DNA ladder of 1200 bp were shown, but no PCR product were detected for bovine DNA and the negative control (Fig.1).

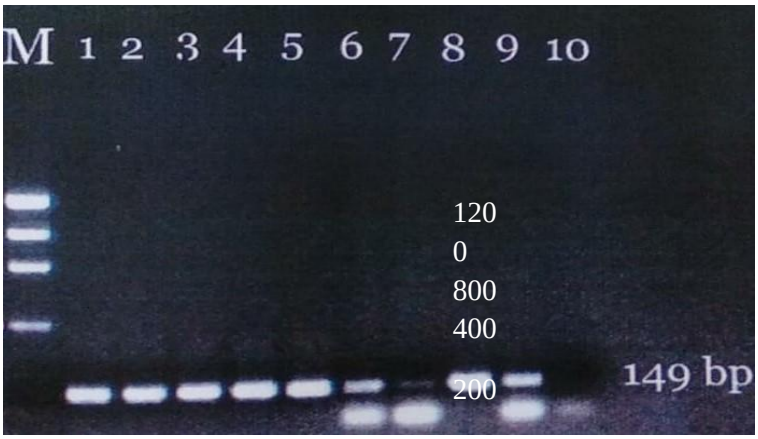

Fig-1: Agarose gel electrophoresis of PCR-product of beef forgery with pork. Lanes M: 1200 bp. DNA ladder (Promega); 1 to 5: porcine DNAs, 6 &7: control of bovine DNAs, 8&9: positive control of porcine DNAs, 10: a negative water control.

This result suggested that the processed products circulating in some area in Bali, West Nusa Tenggara and East Nusa Tenggara were safe, free from genetic material originated from porcine and rodent thus safe from counterfeiting with the addition of pork and rat meat in the products tested. Unfortunately, a box of sample that was originally assumed to be beef was faked containing pork. This data further proof physical texture and a specific smell of the tested sample which was strongly suspected to be pork by the meat distributor. Location, type and number of samples of adulteration of pork and rat meat using the PCR method as shown in Tables 2.

Table-2: PCR test results on the 84 DNA samples tested during the surveillance.

Years	Provinces	Sampling places	Type of samples	Total	PCR test
2018	Bali	Retail/market	Meatball	34	Negative
	West Nusa Tenggara	Retail/market	Meatball	10	Negative
	Eastern Nusa Tenggara	Retail/market	Meatball	10	Negative
2019	Bali	Food distributor	Meatball	12	Negative
		Meat storage	Sausages	8	Negative
		Meat distributor	Fresh beef	1	Positive*
	Eastern Nusa Tenggara	Cold storage	Meatball	4	Negative
		Cold storage	Sausages	6	Negative
Total				85	

Note *: 1 box sample of fresh beef passively sent to Disease Investigation Center Denpasar.

DISCUSSION

Lately, there has been often meat counterfeit in processed foods, which raises public concerns about the dishonesty of the products being marketed. Understanding adulteration and detecting the presence of unwanted meat species in processed food or in freshly looking meats is glorious mission in food control and food safety. For this reason, the development of PCR technology for the detection of species-specific animal gene have been very use full and widely used (4-11). As previously mentioned, the aim of this study was to prove the possibility of meat adulteration in some processed foods originated from some areas in provinces of Bali and Nusa Tenggara. Our data showed that specific porcine DNAs isolated from pork sample that was previously thought to be beef, were successfully amplified by PCR with amplicon size of about 149 bp. Although the number of samples tested was limited (1 box), referring to Figure 1, the PCR product was specific and similar to that reported by [12] and [10], and no primer dimer was observed. This finding conformed the ability of PCR in amplifying a tiny and a specific DNA sample become millions or billions of copies so that can be visualized in agarose gel. It was also considered that the primers sequences provided by [10] and used in this study may slightly cross-reacted with the bovine genes, although the amplicons of bovine DNAs were lest stronger than of porcine origin. A minor cross-reaction of primers with extracted DNA of bovine detected at similar amplicon size of about 149 bp (Fig 1 lanes 6 and 7. This indicated that the primer not only annealed with porcine DNA sequences during amplification step but also annealed with bovine gene sequences. The porcine set of primers may cause a minor false-positive when used to test rodent DNAs. To clarify this condition, the PCR product of the bovine DNA could be further confirmed by sequencing analysis and or be always including a non-porcine DNA control when testing porcine DNA with this primer. The case of adulteration of pork on beef reported in this study may be triggered by beef price which was more expensive and the appearance of pork originated from wild pigs was frequently almost similar to beef. In contrast, however, all of the processed food tested originated from many different locations of sampling were negative PCR, not to contain genetic material of pork and rodent origins. This condition Luckily there has been PCR technology that can save buyers from being scammed and save their health, mainly because food containing pig sources are non halal and unlawful for Muslims community to consume [14].

CONCLUSIONS

Adulteration of meat in processed foods in Bali and Nusa Tenggara Indonesia was not proven by using advance molecular technology and therefore it has been safe from medical aspect in term of not being allergic and associated with halal fatwa for Muslims. A case of pork adulteration on beef founded in this work was a valuable discovery for the future, although currently the sample size was remaining limited. Further similar research should be done routinely and by testing a bigger number of samples to provide a more accurate information for food safety control.

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