VALIDATION OF RFLP-COMBINED PCR TECHNIQUE TO DETECT PORCINE CONTAMINATION IN MEATBALL (HALAL ANALYSIS)

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ABSTRACT

Porcine contamination was one important issue in food certification for Halal. Here, we reported PCR combined with RFLP technique to detect porcine contamination in meatball. Various concentration of pork was added to beef in preparation of the meatball. Mithochondrial DNA was then isolated from the meatball followed by PCR using published primers that amplified cytB gene. A ± 350 bp DNA fragment was resulted from PCR. PCR product was targeted for digestion by means of BamHI and BseDI enzymes. Since these enzymes cut porcine cytB gene but does not cut beef cytB gene, all meatball contain porcine contamination give small DNA fragment (± 220 and 130 bp) in electrophoresis analysis, while the absent of these DNA fragment leading to conclusion that meatball sample free from porcine. This method has been validated to detect up to 1% of porcine present in the meatball.

Keywords: porcine, halal, PCR, RFLP

INTRODUCTION

One of the major issue in the field of food safety is determination of food authenticity (authentication) and forgery detection [1-3]. In Moslem society mixing food products either meat or non-meat products with ingredients such unlawful oil and pork becomes issues that often develops since it related to the halalness of the foods. Technology is needed to justify the food contamination by porcine and necessary for the halal certification of status of products.

One approach to detect porcine contamination in food is by means of DNA test [4]. Several techniques has been developed regarding meat contamination, such as multiplex PCR assay [5], PCR-based finger printing [6] and real time PCR [7]. The most common method was PCR method combined with digestion with restriction enzyme (PCR-RFLP) [4,8].

PCR-RFLP method for porcine detection can be performed by amplification of gene encode cytochrome b on mtDNA. Several PCR primers for this amplification have been published. One of them amplifies from position 70 to 429 of the gene result in DNA fragment with 359 bp length. Restriction the fragment with BamHI and BseDI result in fragment as shown at Fig. 1 and table 1. From table 1, specific length of DNA fragments, 288, 171 bp for BseDI digestion and 213, 115 bp for BamHI digestion, present only if the digested fragments are porcine DNA or contain porcine DNA. Using mtDNA also gives additional advantage since mtDNA present in high concentration in the meat [9]. Erwanto et al. (2007) [10] has reported the performance this methods to detect porcine contamination in the fresh meat mixture. In case of food product the performance of the method could be different since the additional food materials.

Table 1. Comparison of BseDI and BamHI fragment size of 359 bp cytB DNA fragment from various organism

<table>
<thead>
<tr>
<th>CytB fragment, 359</th>
<th>BseDI (bp)</th>
<th>BamHI (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>320,39</td>
<td>328,31</td>
</tr>
<tr>
<td>Sheep</td>
<td>320,39</td>
<td>328,31</td>
</tr>
<tr>
<td>Chicken</td>
<td>328,31</td>
<td>213,115,31</td>
</tr>
<tr>
<td>Pig</td>
<td>288,171</td>
<td>213,115,31</td>
</tr>
</tbody>
</table>

The paper report the performance of the methods in detect porcine contamination in
meatball, a food product consists of meat and flour. The performance tested included specificity and limit detection of the methods.

**EXPERIMENTAL SECTION**

**Materials**
Beef and pork were purchased from the local market in Yogyakarta. DNA isolation was performed by means of NucleoSpin® Food Kits (Clontech). PCR was employed by Illustra™puReTaq Ready-To-GoPCR Beads (GE Healthcare) with primer forward 5’-CCATCAACATTTCATCATGATGAAA-3’ and primer reverse 5’-GCCCTTCAGAATGATATTTGTCCTCA-3’ synthesized by 1stBASE. Enzymes BseDI and BamHI were from Fermentas.

**Instrumentation**
Main instrument for this experiment was PCR machine GeneCycler 10432 (Biorad), and set of electrophoresis apparatus for gel agarase electrophoresis.

**Procedure**

**DNA Isolation from Meatball**
The meatball with various concentration of porcine meat was prepared according to common cooking recipes. DNA isolation was performed according to kits booklet (GE,2007) with a minor modification. About 200 mg of meatballs were homogenized with a mortar and pestle under liquid nitrogen. The powdered, homogenized tissue were then transferred to microcentrifuge tube and extracted with of Buffer CF helped by Proteinase K by incubation at room temperature 24 hrs. The next steps were carried out exactly according to kits booklet. The isolated DNA was checked by agarose gel electrophoresis and estimated its concentration by means of UV spectrophotometer.

**PCR of cytB DNA Fragment**
PCR reaction was started by adding approximately 100 ng isolated DNA, 10 pmole of each forward and reverse primer to PCR bead tube. The tube contents were then mixed by gently flicking the tube with a fingers followed by vortex and short centrifugation. The tubes were then put into thermalcycler machine and programmed for 30 cycles reaction with condition: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After last cycle an extra extension cycle was performed for 10 min. The PCR product was checked with agarose gel electrophoresis and estimated its concentration by means of UV spectrophotometer.

**Digestion with BseDI and BamHI**
For BamHI digestion, 0.5 ng PCR fragment was mixed with 2 µL BamHI buffer 10x, 20 U of BamHI enzyme and added with nuclease free water to final volume 20 µL. The mixture was incubated at 37 °C for 24 hrs. For BseDI digestion 0.5 ng PCR fragment was mixed with 2 µL BseDI buffer 10x, 20 U of BseDI enzyme and added with nuclease free water to final volume 20 µL. The mixture was incubated at 55 °C for 24 hrs.

**RESULTS AND DISCUSSION**

**Specificity of the Method**
Two type of meatball, free-porcine meatball (beef meatball) and pork meatball were used for specificity test. The methods will categorized as specific if no interference in the RFLP result, meaning typical restriction fragment (288, 171 bp for BseDI digestion and 213, 115 bp for BamHI) only present on pork meatball but not on free-porcine meatball. Figure 2 shows electrophoresis result of PCR of both meatball. The size of the fragments is same at 359 bp.

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The product of digestion of PCR fragment with digestion enzymes was shown at Figure 3. The result shows that typical restriction fragment for porcine only present on pork meatball and not present at beef meatball. Based on this result, the method has good specificity porcine in the meatball.
Limit of Detection

Since the present of the porcine in the meatball could be coincident i.e. due to preparation of pork meatball and beef meatball using same equipment, the limit detection of the method become important. The contamination of porcine up to 1% was tested, since in most cases pork is added to meatball in purpose, so the 1% value is more than enough to overcome the problem.

PCR of meatball sample with various porcine contamination result in same fragment at 359 bp, as shown at figure 4. Restriction of the PCR fragment gives no typical porcine fragment for negative control (beef meatball). However, meatball sample with only 1% pork on meat component gives as clear as positive control (pork meatball) for the present of porcine typical fragment. The results are shown at Figure 5. Based on the result the method could still detect porcine contamination up to 1% and possibly lower that 1% since the digestion result of 1% porcine contaminated sample still gives clear porcine typical fragment.
Application of the Method to test commercial meatball

Samples from three well known meatball restaurant in Yogyakarta were tested. PCR result and RFLP analysis of the samples are shown at Figure 6 and Figure 7, respectively. Based on the result it can be concluded that the samples from the restaurant are free from porcine contamination although none of them is halal certified.

CONCLUSION

PCR of cytB fragment followed by RFLP analysis using BseDI and BamHI restriction enzymes could effectively detect porcine contamination in meatball with high specification. The capability of the method has been tested up to 1% contamination. The method has also been applied to test sample of commercial meatball result in no contamination even in the non certified meatball.

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REFERENCES