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Pig species identification in meatballs using polymerase chain reaction- restriction fragment length polymorphism for *Halal* authentication

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Abstract: The detailed information on the chemical and nutritional content is essential for consumers in choosing meat-derived food products. For moslem communities, it is prohibited to consume pork-contained or other pig derivatives foods. Unfortunately, meat adulteration by means of mixing beef and chicken with pork or other pig derivatives frequently occurs in the market. This habits cause difficult identification of beef and chicken that are free from pork and other derivatives products. Genomic DNA of pig, bovine, and chicken were isolated and subjected to PCR amplification targeting the mitochondrial cytochrome b gene. Pig species differentiation was determined by digestion of 359 bp amplified product obtained with *BseDI* restriction enzymes, which generated pig species electrophoresis pattern. PCR-Restriction Fragment Length Polymorphism (RFLP) revealed the presence of pork in meatball product which can be distinguished among bovine, chicken, and pig samples. Pig mitochondrial cytochrome DNA gene was cleaved into 228 bp and 131 bp fragments but the bovine, and chicken cytochrome b gene were not digested by *BseDI* enzyme. PCR-RFLP technique using *BseDI* restriction enzymes is reliable for the detection of pork in meatball for the *Halal* authentication.

Key words: Pig species, Identification, PCR-RFLP, *Halal* authentication

Introduction

Indonesian traditional meatballs or known as "bakso" is one of the comminuted meat products and gains the popularity among all classes of Indonesian society. The products are served in hot soup with other stuffs such as tofu, noodle, cabbage and chili or tomato sauce. Meat used to make *bakso* originally comes from beef, but nowadays some others such as chicken, fish, and pork are also commonly used in some meatball products (Purnomo and Rahardiyan, 2008). The wide variety of meatball products availabe on the market in Indonesia seems favourable but leads to several fears for Muslim community, because the presence of pork in meatball products are prohibited to be consumed (Rohman *et al*., 2011). This is an important challenge for the people in charge of the official control of food which have an obligation to to verify the species of meat ingridients that are not always easily identifiable.

The strategies used to detect the adulterated products have traditionally relied on wet chemistry to determine the amount of a marker compound or compounds in a test material followed by a comparison of the value(s) obtained with those previously documented for authentic material of the same type. This approach is often time-consuming and therefore expensive; therefore, some analytical methods offering fast and reliable results are continuously developed by some researchers (Downey, 1998). One of them is DNA-based methods.

Many various methods based on DNA techniques have developed such as multiplex PCR assay (Matsunaga *et al*., 1999) and PCR-based finger printing (Saez *et al*., 2004). Colgan *et al.* (2001) analyzed meat bone meal using real time PCR to investigate the meat source origin and to verify the quantity of meat in DNA mixture complex. Lopes-andreo *et al.* (2005) also studied meat species identification using the same methods. Similarly, identification of the added por or porcine in a mixture of meat products can be carried out based on the identification of porcine DNA. Therefore, the aim of this study was to apply the PCR-RFLP technology as a tool for meat species identification on samples of the Indonesian meatballs.

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Materials and Methods

Sample preparation and DNA extraction

Authentic muscle samples of beef, pork and chicken were obtained from the traditional market in Yogyakarta, Indonesia. Meatball was prepared in laboratory scale with separate equipment to prevent cross contamination. Meatball samples were prepared by mixing pork with beef or chicken at a final concentration of pork at 0; 1.0; 2.5; 5.0; 10.0 and 25.0 % (w/w).

DNA was extracted from meatball samples using the *High Pure PCR Template* protocol for animal tissue provided with the *High Pure PCR Template Kit* (Roche, Germany). Approximately 50-100 mg of meatballs was blended using a commercial blender and placed in a 1,5 ml microcentrifuge tube. A-100 µl of *tissue buffer* and 40 µl *Proteinase* K were added and mixed by vortexing. The mixture was incubated at 55°C in a water bath overnight to disperse the sample until the tissue was completely lysed. The samples were then added with 200 µl binding buffer and incubated at 70°C for 10 min. The mixture was mixed b vortexing for seconds, added with 100 µl isopropanol, mixed vigorously and placed high filter tubes. The samples was subsequently poured in the collection tube, placed in table top centrifuge, and spun at 8,000 g for 1 min. The flow-through and *collection tube* were discarded and the *High Filter Tube* was placed in a new 2 ml *collection tube*. A-500 µl of *wash buffer* was added and spun at 8,000 g for 1 min. The flow-through and collection tube were discarded and the *High Filter Tube* was placed in another 2 ml *collection tube*. The high filter tube was dried by centrifugation for 10 seconds, and the supernatant flow-through was discarded. The *High Filter Tube* was placed in a clean 1.5 ml micro centrifuge tube. A-200 µl of *pre-warmed elution buffer* was added and spun at 8,000 g for 1 min to elute. The DNA solution was stored at 4 °C.

PCR amplification of a conserved Cytochrome 2b of Mitochondrial gene fragment

The set of primers used for amplification consisted of Cytb-FW and Cytb-REV oligonucleotides as follows: CYT b FW 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3', CYTb REV 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'. Amplification of the mt cyt b gene was performed in a final volume of 25 µl containing 250 ng of extracted DNA, mega-mix royal (optimized mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in 2 X reaction buffer (6 mM $MgCl_2$ with 400 µM dNTPs, stabilizer and blue loading dye) (Microzone Ltd, West Sussex, UK), and 20 pmol of each primer. Amplification was performed with a thermal cycler according to the following PCR step-cycle program: pre-denaturation of 94°C for 2 min to completely denature the DNA template, followed by 35 cycles of denaturation at 95°C for 36 s, annealing at 51°C for 73 s, and extension at 72°C for 84 s. Final extension at 72°C for 3 min followed the final cycle for complete synthesis of elongated DNA molecules. Two microlitres of PCR products were electrophoresed at constant voltage (50V) on 2% agarose gel (Promega, Madison, USA) for about an hour 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.

Restriction fragment length polymorphism

Two units/µl of RE *BseDI* (Fermentas) were applied to 10 µl of amplified DNA in a final volume of 20 µl digestion mixture [containing 1x reaction buffer (10 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 0,2 mg/ml BSA, 1 mM DTT and 50% glycerol)] and were incubated at 55°C for 3 h for optimal result. A-5 µl of the digested samples were electrophoresed at constant voltage (50 V) on 2% agarose gel (Promega, Madison, USA) for about an hour in 1x TBE buffer, pH 8.0 and stained by ethidium bromide. A-100 bp (Promega, Madison, USA) was used as size reference. The gel photo was taken using the Syngene gel documentation system.

Results and Discussion

PCR based amplification was carried out based on the sequence of the mitochondrial cytochrome b of the products. For restriction fragment length polymorphism was carried out by digesting the PCR products using BseD I enzymes. Genomic DNA isolation from the meatball can be extracted with this kit, but it is ascribed to the fact that thermal strongly accelerates DNA degradation from the meatball samples (Figure 1). The data was in comfort with the finding of Arslan *et al.* (2006) and Tanabe *et al*. (2007) who reported that heating of the samples by various treatment did not significantly affect the DNA and it was able to detect. Matsunaga *et al*. (1999) has also studied of DNA isolation in meat which was processed with high temperature around 100 and 120°C for 30 min of various meat flesh such as cattle, goat, chicken, sheep, horse and pig, while Tanabe *et al.* (2007) provided similar data of pork at various cooked. According to Martinez and Yman (1998) and Saez *et al.* (2004), the heat treatments

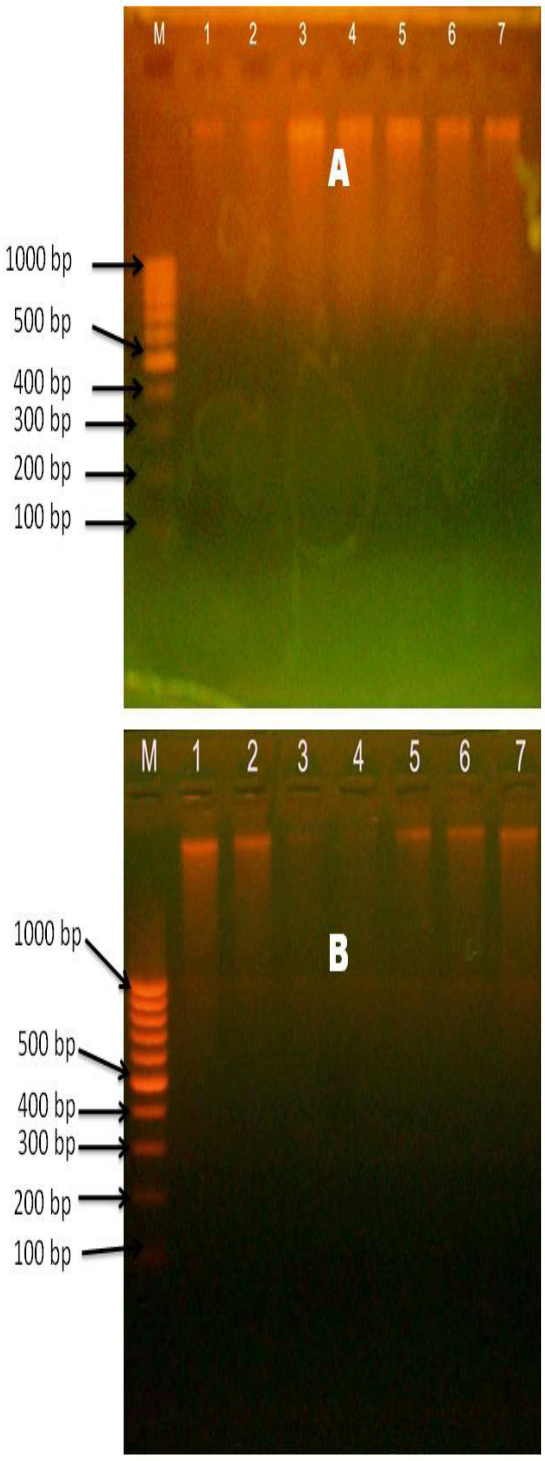

Figure 1. Total genomic DNA extracted from beef-pork meatball and chicken-pork meatball. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (beef 75% : pork 25%) 3: (Beef 90% : Pork 10%), 4: (Beef 95% : Pork 5%)5: (Beef 97% : Pork 3%), 6: (Beef 99% : Pork 1%), 7: (Beef 100 %). (B): M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (chicken 75% : pork 25%) 3: (chicken 90% : Pork 10%), 4: (Chicken 95% : Pork 5%)5: (Chicken 97% : Pork 3%), 6: (Chicken 99% : Pork 1%), 7: (Beef 100 %).

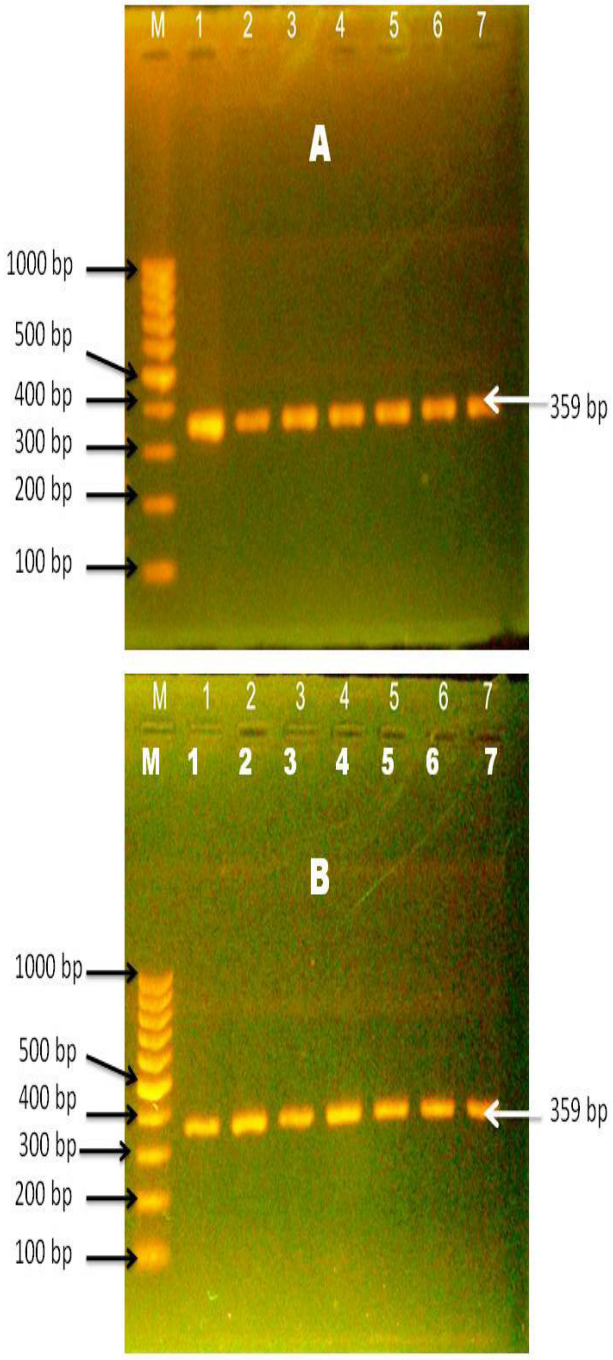

Figure 2. PCR products of cytochrome b gene fragments 359 bp long of samples from different meatballs product separated by 2% high-resolution agarose gel electrophoresis. PCR amplification using cyt b universal primer. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (beef 75% : pork 25%) 3: (Beef 90% : Pork 10%), 4: (Beef 95% : Pork 5%)5: (Beef 97% : Pork 3%), 6: (Beef 99% : Pork 1%), 7: (Beef 100 %). (B): M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (chicken 75% : pork 25%) 3: (chicken 90% : Pork 10%), 4: (Chicken 95% : Pork 5%)5: (Chicken 97% : Pork 3%), 6: (Chicken 99% : Pork 1%), 7: (Beef 100 %).

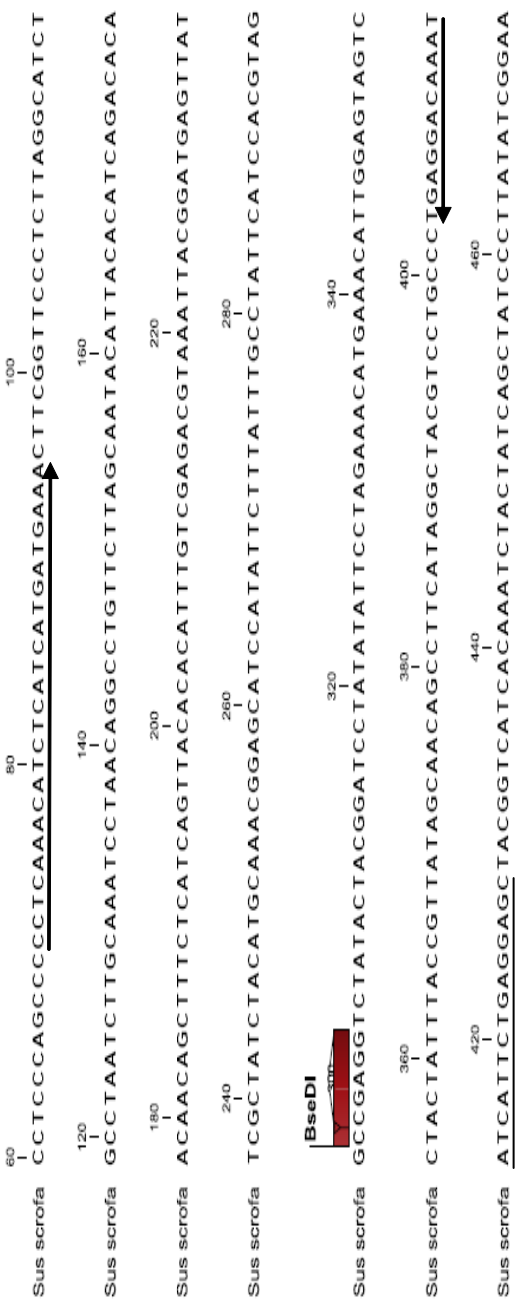

Figure 3. Sequences of nucleotide *cytochrome b* of *Sus scrofa* (pig) restriction site by *Bse*DI using CLC Sequencing Software and universal primer position in the fragment of *cytochrome b* gene (→ dan ←).

Genomic DNA was applied as a template for the PCR amplification using universal primers. Gene of cytochrome *b* was selected for the PCR amplification and resulted a DNA fragment of approximately 359 bp (Figure 2). This result indicated that isolated DNA of mixture meatball was enough for PCR amplification. The same result of PCR amplification has also been reported previously (Kocher *et al.,* 1989; Aida *et al*., 2005; Erwanto *et al*., 2011). The selection of target gene and primers affecting sensitivity and specification of method for detection. PCR method was very sensitive when primer target represent a gene multicopy of like gene mitochondrial. This research used the area mitochondrial DNA of the cytochrome *b* as target for detection of porcine.

The PCR reaction allowed fragments of the expected length to be obtained in all meatball samples either beef or chicken mixed with pork, although with various efficiencies. The mitochondrial cytochrome b gene was selected in this study as template for DNA amplification, because it has an acceptable length and an adequate grade of mutation and there are numerous sequences available in the DNA bank databases (Kocher *et al*., 1989). The mitochondrial primers Cyt b-FW and Cyt b-REV was able to amplify a conserved 359 bp region of the cytochrome b gene of all animal studied, namely chicken, beef and pork.

Sequence DNA of cytochrome b gene of cattle, goat, chicken and pig obtained from database of NCBI was further employed for sequence alignment using software of CLC sequencer. The similarity of the mitochondrial cytochrome b gene among beef, mutton, chicken and pork was 86.64%. As a result of the preliminary CLC sequencer software analysis for the detection of specific restriction sites on pig sequence, a site recognized by *BseDI* enzyme was cleaved into two fragments, namely 131 bp and 228 bp (Figure 3). Based on RFLP pattern using CLC sequencer, *Bse*DI was applicable to differentiate or identify among four species.

The digestion of PCR products resulted the different fragment sizes, it was 131 and 228 bp at PCR product of porcine. Basically, PCR product of mutton could also be digested, but DNA length size was very short (approximately 5-20 bp), consequently, it could not be seen at 2% agarose gel (Figure 4). A clear band with a length between 100 and 150 bp was observed and thus referable to the 131 bp fragment, as shown in Figure 4 (lane 1). In the same lane, a thicker band can be traced back to the 228 bp fragment.

The data obtained suggests that compared with *BsaJI* endonuclease profiles, the DNA restriction patterns obtained after digestion of the amplicons with *BseDI* enzymes consisted of same patterns.

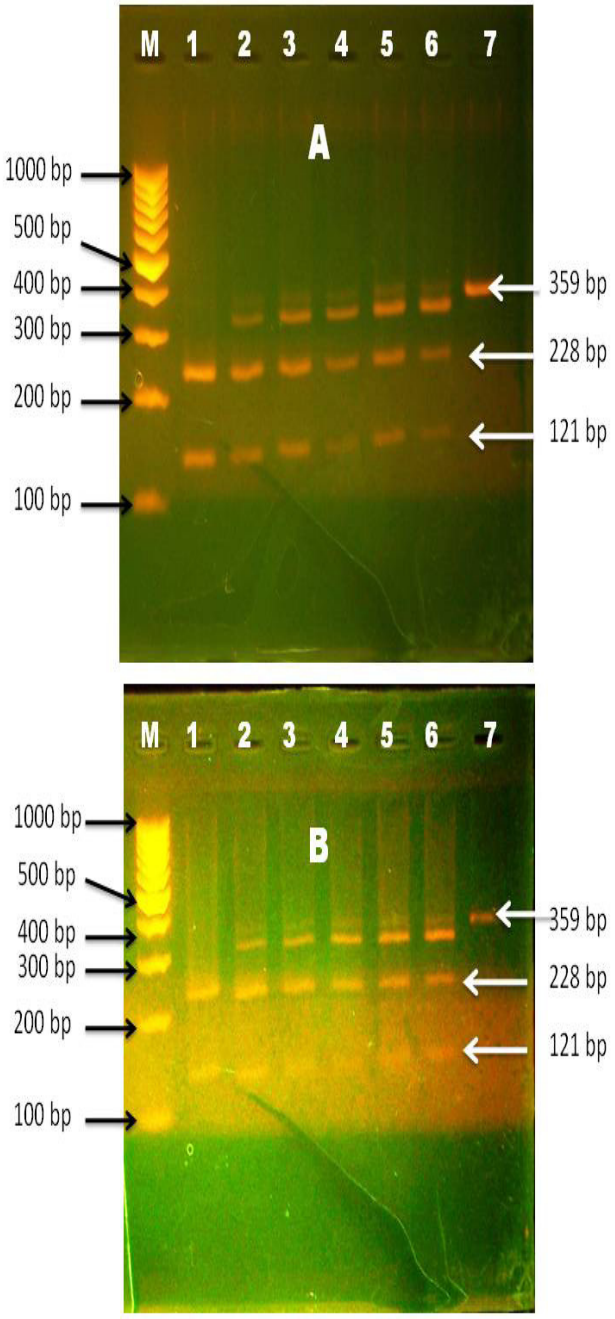

Figure 4. Restriction fragment produced by *BseDI* restriction enzyme on 359 bp amplicons of cytochrome b gene from different meatball products separated by 2% high-resolution agarose gel electrophoresis. PCR amplification using cyt b universal primer. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (beef 75% : pork 25%) 3: (Beef 90% : Pork 10%), 4: (Beef 95% : Pork 5%)5: (Beef 97% : Pork 3%), 6: (Beef 99% : Pork 1%), 7: (Beef 100 %). (B): M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (chicken 75% : pork 25%) 3: (chicken 90% : Pork 10%), 4: (Chicken 95% : Pork 5%)5: (Chicken 97% : Pork 3%), 6: (Chicken 99% : Pork 1%), 7: (Beef 100 %).

The difference between *BsaJI* and *BseDI* restriction enzyme is the incubation time for the digestion. *BseDI* needed 3 h for digestion, while *BsaJI* enzyme needed more than 12 h (Aida *et al*., 2005).

PCR amplification of cytochrome b gene followed by digestion by BseDI restriction enzymes was a powerful technique for the identification of pork or other pig derivative products contamination, due to its simplicity and sensitivity. The cytochrome b gene alignment using CLC sequencer software showed that pig intra species have the same restriction sites and their homology was 98.2%.

Conclusions

Our results allow us to conclude that PCR-RFLP of the mitochondrial Cytochrome b gene is a suitable alternative technique that can be applied to the detection of pig species present in the commercialized food products such as meatballs.

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