Pig Species Identification in Meatballs Using Polymerase Chain Reaction Restriction Fragment Length Polymorphism

Y. Erwanto[1], M.Z. Abidin[1], A. Rohman[2] and Sismindari[3]
[1]Division of Animal Products Technology, Faculty of Animal Science, Gadjah Mada University, Yogyakarta
[2]Faculty of Pharmacy, Gadjah Mada University, Yogyakarta
[3]Integrated Laboratory of Research and Testing, Gadjah Mada University, Yogyakarta

ABSTRACT

The information given to consumers is essential for them to choose one food product over another. The falsification of food contents on product labels is a widespread problem, especially with products related with pig or others prohibited food in Islam. Proving conclusively that fraud has occurred requires the detection and quantification of food constituents. Falsifications of meat or food are often biochemically similar to the materials they replace, consequently the identification and measurement extremely difficult. The DNA based methods have now been successfully adapted for detection of food substitution. In this research, Polymerase Chain Reaction (PCR) products of cytochrome b mitochondrial DNA gene were applied to identify the existence of pig in meatball product. 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 obtained 359 bp amplified product with BseDI restriction enzymes, which generated pig species electrophoresis pattern. PCR-Restriction Fragment Length Polymorphism (RFLP) revealed the presence of the pig meat in meatball product and distinguished between bovine, chicken, and pig sample. 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. The digestion was conducted at 55^{o}C for 3 h and visualization of the digest product was performed in 2% agarose gel. PCR-RFLP technique using BseDI restriction enzymes is reliable for the detection of the pig meat in meatball for the *Halal* authentication.

Key words: pig species, identification, PCR-RFLP, halal authentication

INTRODUCTION

Indonesian traditional meatballs is one of the comminuted meat products and its popularity in all classes of Indonesian society. The products are served in hot soup with others stuff such as tofu, noodle, cabbage and chili or tomato sauce and the popular name in Indonesia is called bakso. Meat are processed to make bakso originally from beef but nowadays some others such as chicken, fish, and pork commonly also been mixed in some meatball products. The wide variety of meatball products availabe on the market in Indonesia seems favourable but leads to several fears, where almost population are moslem who prohibited to consume pork. This is an important challenge for the people in charge of the official control of food, that have to verify the species of meat ingridients that are not always easily identifiable.

Strategies utilized to detect an adulterated product 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; it also has the shortcoming that food adulterers are becoming increasingly sophis-ticated at masking their efforts and the range of analytes which must be quanticed to ensure authenticity is continuously increasing (Downey, 1998).

Many various methods based on DNA techniques have developed such as multiplex PCR assay (Matsunaga et al., 1999), *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) was also able to identify the meat species using the same methods.

Processed meat products such as sausage, meatball, chicken nugget and cornet were exposed with the high temperature during meat processing. Some of the heating of meat will degrade the DNA then the isolation of DNA will be difficult. The adulterated of porcine in mixture of processed meat products can be identified with the method which is based on DNA. Although high temperature treatment affects the quality DNA, did not make the constraint in identifying of porcine material at processed meat.

However the identification of meat species in Indonesia was very rare and there was a little publication on this field. This study reported PCR-RFLP method for meat species identification in Indonesian meatball using DNA mitochondrial as universal primer in PCR reaction. The digestion of the PCR amplicon products to determined meat species was established by BseDI restriction enzyme.

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 unexpected cross contamination. Test meatballs were prepared with pork meat added to the final concentration of pork were 0; 1; 2, 5; 5; 10 and 25 percent to beef or chicken meatballs.

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 meat samples was blended using a blender, placed in a 1.5 ml microcentrifuge tube. Three hundred microlitres *tissue buffer* and 40 µl *Proteinase* K were added and mixed by vortexing. The mixture was incubated at 55°C in a water bath to disperse the sample overnight until the tissue was completely lysed. The following day, 200 µl of *binding buffer* was added and incubated for 10 min at 70°C. The mixture was mixed by vortexing for 15 s. One hundred microlitres isopropanol was added to the sample, mixed thoroughly by vortexing. Put into the *High Filter Tube* in *collection tube*, and then pour sample in it. Placed all tube in table top centrifuge and spun at 8,000 g for 1 min. The flow-through and *collection tube* was discarded and the *High Filter Tube* was placed in a new 2 ml *collection tube*. Five hundred microlitres *wash buffer* was added and spun at 8,000g for 1 min. The flow-through and collection tube was discarded and the *High Filter Tube* was placed in another 2 ml *collection tube*. After throw out solution, then spun at full speed for 10 s to dry the *High Filter Tube* and the flow-through and *collection tube* was discarded. The *High Filter Tube* was placed in a clean 1.5 ml micro centrifuge tube. Two hundred microlitres *pre-warmed elution buffer* was added and spun at 8,000g 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 Cyt b-FW and Cyt b-REV oligonucleotides:

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 5°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 (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 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 *Bse*DI (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. Five microlitres 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

This research was aimed to develop a specific and sensitive method to identify pork meat species. PCR-RFLP based on the sequence of the mitochondrial cytochrome *b* gene by using a pair primer was used as an analytical tool for pork meat identification. The PCR amplicon then digested using BseDI restriction enzyme to distuingesed of chicken, beef and pork.

DNA was extracted from meatball in various meat ingridients using the High Pure PCR Template Kit (Roche). DNA extraction was done using the proteinase K 1 mg/ml. Genomic DNA isolation from the meatball can extracted with this kit but it is ascribed to the fact that thermal strongly accelerates DNA degradation from the meatball samples (Figure 1). This result coincided to Arslan et al. (2004) and Tanabe et al. (2007) that heated process by various treatment did not significantly affected to DNA fragment detection. 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 minutes of various meat flesh as cattle, goat, chicken, sheep, horse and pig, while Tanabe et al. (2007) provided similar to process of pork at various cooked. According to Martinez and Yman (1998) and Saez et al. (2003) reported that heat treatments which mainly affected the quality DNA causing degradation into small size fragment.

Genomic DNA was used as a template for the amplification of PCR with the universal primer. Gene of cytochrome *b* used for the amplification of PCR resulting fragment of approximately 360 bp (Figure 2.). This result indicated that isolate DNA of mixture meatball was enough for amplification on PCR reaction. The same result of amplification has also been reported yet, according to Kocher et al. (1989), Aida et al. (2005) and Erwanto et al. (2007).

Selection of target gene and primer design very influenced sensitivity and specification of method of 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.

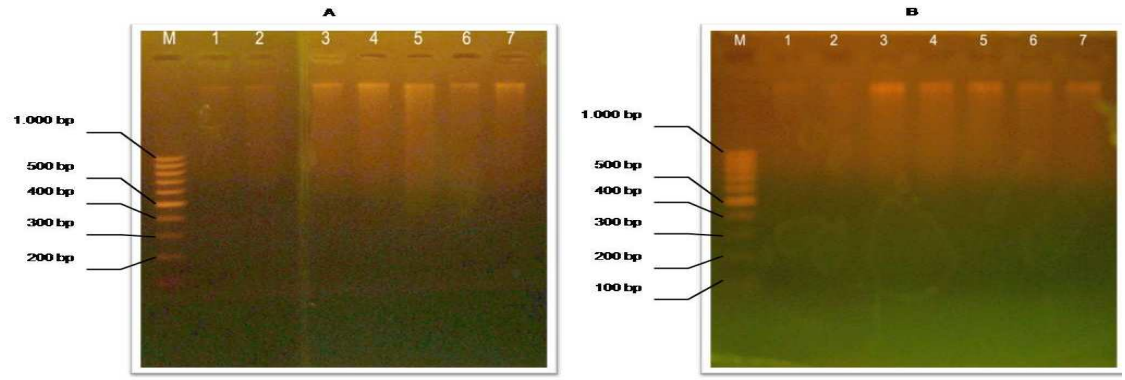

Figure 1. Total genomic DNA extracted from beef-pork meatball and chicken-pork meatball. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (beef 75% : pork 25%) 3: (Beef 90% : Pork 10%), 4: (Beef 95% : Pork 5%)5: (Beef 97% : Pork 3%), 6: (Beef 99% : Pork 1%), 7: (Beef 100 %). (B): M: marker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

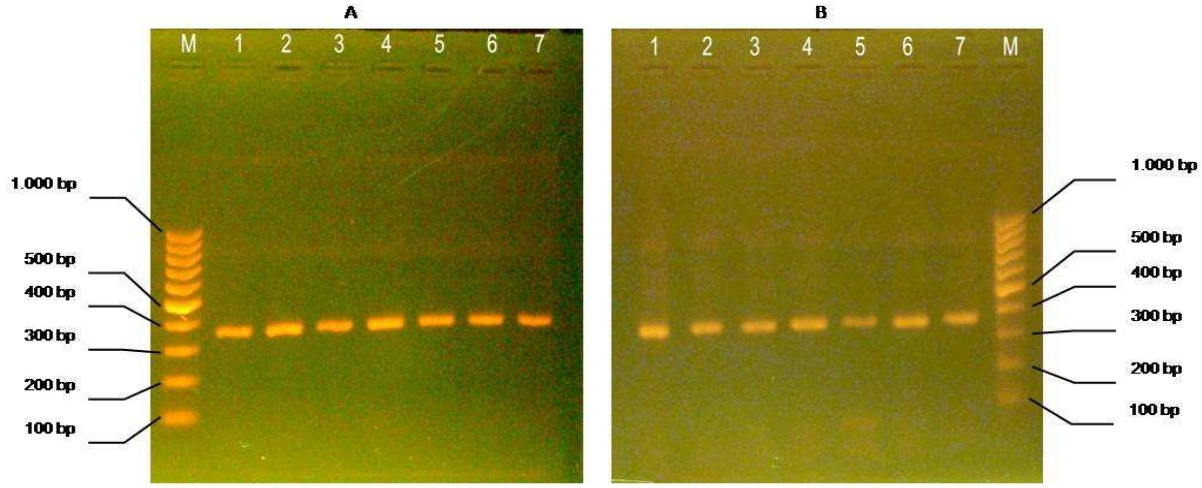

Figure 2. PCR products of cytochrome b gene fragments 359 bp long of samples from different meatball products separated by 2% high-resolution agarose gel electrophoresis. PCR amplification using cyt b universal primer. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (100%), 2: (beef 75% : pork 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 %)

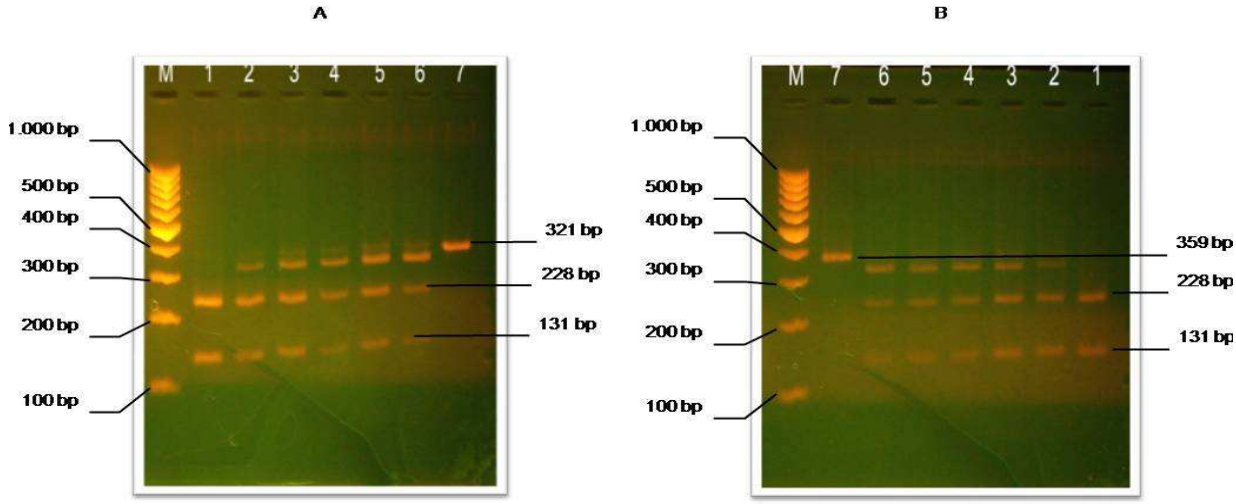

Figure 3. Restriction fragment produced by BseDI restriction enzyme on 359 bp amplicons of cytochrome b gene from different meatball products separated by 2% high-resolution agarose gel electrophoresis. PCR amplification using cyt b universal primer. (A) M: marker 100 bp DNA ladder (Invitrogen), 1: pork (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 PCR reaction allowed fragments of the expected length to be obtained in all meatball samples both beef and chicken mixed with pork, although with variable efficiency. PCR has the potential sensitivity and specificity required to achieve detection of a target sequence from template DNA. The mitochondrial cytochrome b gene has been 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 databases (Kocher et al., 1989).

The mitochondrial primers Cyt b-FW and Cyt b-REV used in the PCR technique developed in this work successfully amplified a conserved 359 bp region from the cytochrome b gene of all chicken, beef and pork individuals analyzed.

Sequence DNA of cytochrome b gene cattle, goat, chicken and pig obtained from database NCBI, then employed by alignment using the software CLC sequencer. The result average of alignment mitochondrial of cytochrome b gene among beef, mutton, chicken and pork is 86.64%.

As a result of the preliminary computerized analysis for the detection of specific restriction sites on pig sequence, a site recognized by BseDI enzyme was cleaved into two fragment 131 bp and 228 bp long were consequently expected. A clear band with a length between 100 and 150 bp and thus referable to the 131 bp fragment can be observed in Fig 3 (lane 1). In the same lane a thicker band can be traced back to the 228 bp fragment.

The results obtained suggest that, compared with BsaJI endonuclease profiles, the DNA restriction patterns obtained after digestion of the amplicons with *BseD*I enzymes consisted of same patterns. The difference between BsaJI and BseDI restriction enzyme is the incubation time for the digestion. Using BseDI needed 3h for digestion but digestion using BsaJI have to incubate for more than 12h.

Based on the laboratory analysis RFLP method can preliminary observed the DNA fragmentation using CLC sequencer software. The result of cytochrome b alignment using CLC sequencer softwer showed that pig intra species have the same restriction sites and their homology was 98.2%.

CONCLUSION

PCR – RFLP of the mitochondrial Cytochrome b gene is a suitable alternative that can be applied to the detection of pig species present in commercialized products such as meatballs.

ACKNOWLEDGEMENTS

This research was financially supported by grants from Riset Unggulan Strategis Nasional LPPM-UGM (Grant number LPPM-UGM/1309 /2009) and fully supported by Inte-grated Laboratory of Research and Testing, Universitas Gadjah Mada.

REFERENCES

Aida, A.A., Y.B. Che Man, C.M.V.L. Wong, A.R. Raha, R. Son. 2005. Analysis of raw meats and fats of pigs using polymerase chain reaction for Halal authentication. Meat Science. 69, 47–52.

Arslan A., O.I. Ilhak, and M. Calicioglu. 2006. Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat Science. 72, 326–330.

Colgan S., L. O'Brien, M. Maher, N. Shilton, K. McDonnell, and S. Ward, 2001. Development of a DNA-based assay for spesies identification in meat and bone meal. Food Research International 34/5, 409-414.

Downey, G. 1998. Food and food ingredient authentication by mid-infrared spectroscopy and chemometrics. Trends in analytical chemistry, vol. 17, no. 7, p:418-424.

Erwanto, Y., Arief. B. W., and Rusman. 2007. Identifikasi daging babi dengan PCR-RFLP sebagai acuan untuk menentukan status kehalalan. (Pig Meat Identification using PCR-RFLP to determine the Halal Status). Proceeding Seminar Cluster, LPPM, Universitas Gadjah Mada.

Kocher, T. D.; Thomas, W. K.; Meyer, A.; Edwards, S. V.; Pa¨a¨bo, S.; Villablanca, F. X.; Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. U.S.A. Vol. 86. 6196-6200.

Lopez-Andreo, I., L. Lugo, A. Garrido-Pertierra, M. I. Prieto, and A. Puyet. 2005. Identification and quantitation of spesies in complex DNA mixture by real-time polymerase chain reaction. Analytical Biochemistry. 339.p. 73-82.

Matsunaga, T., K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada, Y. Shinmura. 1999. A quick and simple method for the identifcation of meat species and meat products by PCR assay. Meat Science. 51. 143-148.

Martinez and M. I. Yman. 1998. Species identification in meat product by RAPD analysis. Food Research International. 31. 459-466.333.

Saez, R. Y. Sanz and F. Toldra. 2003. PCR-based fingerprinting technique for rapid detection of animal species in meat product. Meat Science. p, 659-665.

Tanabe, S., Eiji M., Akemi M., and Kazuhiro M. 2007. PCR method of detecting pork in foods for verifying allergen labeling and for identifying hidden pork ingredient in processed foods. Bioscience Biotechnology Biochemistry. 71. pp : 70075-1-5.