International Food Research Journal 23(1): 370-374 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

# Analysis of pork contamination in *Abon* using mitochondrial D-Loop22 primers using real time polymerase chain reaction method

[1,2]Rahmawati, [1]Sismindari, [3,4]Raharjo, T.J., [1*]Sudjadi and [1,3]Rohman, A.

*[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta 55281, Indonesia*
*[2]Universitas Muslim Indonesia, Makassar, Indonesia*
*[3]Research Center of Halal Products, Gadjah Mada University, Yogyakarta 55281 Indonesia*
*[4]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Yogyakarta 55281 Indonesia*

**Article history**

*Received: 19 September 2014*
*Received in revised form: 26 May 2015*
*Accepted: 19 June 2015*

**Keywords**

*Shredded meat (abon)*
*Mitochondrial D-loop22*
*Polymerase chain reaction*
*Halal authentication*

**Abstract**

The presence of pork in any meat products including abon (shredded meat) is not allowed for Islamic community. Therefore, analysis of porcine is very essensial. One of the specific and sensitive methods for such analysis is DNA-based methods using polymerase chain reaction (PCR). The analysis was carried out by performing porcine DNA amplification using primers designed based on the D-loop mitochondrial region of pig. The primers can amplify the target DNA at 22$^{nd}$-197$^{th}$ basal orders with the amplicon of 176 pb. The specificity test was done on fresh tissues of pig, cow, chicken, goat and horse. The shredded meat was prepared from the mixture of pork- beef, with the concentrations of pork are 0.5; 1; 3; 5 and 10%. The detection limit test was done on 100% shredded pork (with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg) and on the mixed shredded meat of pork-beef (with the concentrations of 0.5; 1; 3; 5 and 10% of pork). The repeatability test was done on 100% shredded pork and the mixed shredded meat of pork-beef with the same concentrations above. The results showed that the mitochondrial D-loop22 primers can specifically detect the target pig DNA on both fresh tissues and the mixed shredded meat of pork-beef. The detection limit of pig DNA on the shredded pork was 10 pg, and 0.5% for the mixed shredded meat of pork-beef. Coefficient of variation (CV) for repeatibility on shredded pork was 16.28%, while that on the mixed shredded meat of pork-beef was 7.46%. The analysis of pig DNA on shredded beef in market did not show the presence of DNA amplification.



## Introduction

In the last several years, the identification of animal species has been a main attention because of increasingly high awareness in society on both food substance or composition and food labeling systems containing the contents of the product (Soares *et al*., 2013). It is closely related to food safety, i.e. the issues of food product authentication and forgery, particularly the forgery using pork and its derivatives (Murugaiah *et al*., 2009; Martin *et al*., 2009; Koppel *et al*., 2011). It causes controversial reactions in society because pork is clearly prohibited for the Islamic community, many people are highly hypersensitive to pork, and there are vegetarian life styles. Therefore, a method should be developed to specifically and selectively identify the pork (Kesmen *et al.*, 2007; Soares *et al*., 2013).

A lot of methods have been developed to identify of porcine i.e. Capillary Gel Electrophoresis (Hernandez-Chavez, 2011), ELISA (Chan & Hsieh, 2000), Chromatography (Chou *et al*., 2007), Electronic Nose (Nurjuliana *et al*., 2011), FTIR (Rohman *et al*., 2011; Xu *et al*., 2012). Besides, any methods based on DNA identification like Polymerase Chain Reaction-PCR (Aida *et al.*, 2005; Sahilah *et al*., 2011), Duplex-PCR (Soares *et al*., 2010), Multiplex-PCR (Koppel *et al.*, 2011) and real time PCR (Dooley *et al*, 2004; Eugster *et al*, 2008; Kesmen *et al,* 2009; Tjondro and Sismindari, 2012; Ali *et al*, 2012; Syahruni *et al*, 2012). The real time PCR method is one of the identification methods using pig DNA that has largely been developed in this decade because it has several advantages, i.e. it is easy and simple, the measurement is quantitative, and it only requires a small sample size (Fraga *et al*., 2006; Che Man *et al*., 2007; Nakyinsige *et al*., 2012).

One of the foods to be the main target of forgery is shredded meat products. Shredded meat is a typical Indonesian food with the raw materials of meat whose processes include the pounding, heating and frying of meat, which can cause DNA fragmentation,

*Corresponding author.
Email: *jadi_farma@ugm.ac.id*
Tel: +62-274-543120

so that the DNA is difficult to identify or the amount is too small to be amplified (Meyer *et al.*, 1994). It is the further target of primers attachment.

The identification of pig DNA using the real time PCR method requires specific primers that can only attach on the target of pig DNA fragment at certain basal order, and furthermore the amplification of the fragment is done. Fatimah (2013) designed a set of primers that can amplify the target of pig mitochondrial D-loop fragment, which was tested at the samples of meatballs made from 100% pork and 100% chicken using the real time PCR method with the optimal temperature of 59°C for primers attachment.

To assure the validity of a new primers, the mitochondrial D-loop22 primers is necessary to be validated by doing the specifity test using fresh tissues from five animal species such as pig, cow, chicken, goat and horse as well as using the mixed shredded meat of pork-beef to prove that the mitochondrial D-loop22 primers can only recognize pig DNA as amplification target, but not other targets, the detection limit test using a series of the dilutions of shredded pork and the repeatability test using shredded pork and the mixed shredded meat of pork:beef.

## Material and Methods

*Primers and samples*

The set of mitochondrial D-loop22 primers designed by Fatimah (2013) :

D-LOOP22 Forward : 5'- TCG TAT GCA AAC CAA AAC GCC -3'

D-LOOP22 Reverse : 5'- ATG CAT GGG GAC TAG CAG TTA -3'

Positive controls were done using the tissues of 100% pork and shredded pork, while negative controls were done using those of 100% cow, chicken, goat, and horse.The mixed shredded meat of mixed pork:beef was made with the concentrations of 0.5; 1; 3; 5 and 10%. The branded abon were got at market in Yogyakarta.

*DNA isolation*

The DNA isolation procedure was done using the modified Sambrook method (Sambrook *et al*., 1989). Amplification of the D-loop22 was performed in a final volume of 20 µL containing 50 ng of extracted DNA, 10 µL of Ssofast Evagreen® supermix (Bio-Rad , USA), 1 µL (10 mmol) of each primer and 6 µL of free nuclease water. The real time PCR step-cycle program : pre-denaturasi at 95°C for 30 s, followed by 30 cycles of denaturation at 95°C for 5 s, annealing at 59°C for 30 s and extention at 72°C for 10 s (Fatimah, 2013).

*Spesificity test*

The specifity test of mitochondrial D-loop22 primers was done in detecting the pig DNA among the DNAs of cow, chicken, goat, and horseas well as in the mixed shredded meat of pork:beef using the real time PCR method. Both detection limit and repeatability test were done using 100% shredded pork (concentrations of 10000, 1000, 100, 10, 5 and 1 pg) and mixed shredded meats of pork:beef (concentrations of 0.5; 1; 3; 5 and 10%).

## Results and Discussion

The mitochondrial D-loop22 primers designed by Fatimah (2013) were validated to examine the validity of primers in detecting the target pig DNA. The validity test included the specificity, limit of detection and repeatability of primers. DNA was obtained from the results of isolation on fresh tissues and the sample of shredded meats using the modified Sambrook method. DNA isolates obtained were analyzed qualitatively by using 0.6% agarosa gel electroforesis and quantitatively by using the UV Spektrophotometer UV of λ 260 and 280 nm, which were useful to find out the content and purity of the DNA isolates.

Temperature optimization for primers attachment was previously done using pig and cow DNAs before using mitochondrial D-loop22 primers for further identification. A range of temperatures for amplification using the real time PCR method was from 50 to 60°C, and the optimal temperature for primers attachment was 59°C. At the temperature, the pig DNA could be amplified maximally with the lowest values of Cq (quantification cycle) , there were no primers dimer and non-specific products as well as the highest value of RFU (ratio of fluorecense unit).

Furthermore, the identification of pig DNA was done using mitochondrial D-loop22 primers with the real time PCR method, including the specifity test of primers on pig DNA from fresh tissues compared with that on the DNAs of cow, chicken, goat, and horse. As a result, D-loop22 mitochondrial primers using the real time PCR method can amplify the pig DNA, but not amplify other DNAs (Figure 1).

The specifity test of primers on the pig DNA from shredded pork (positive control) was compared with that of cow DNA from shredded beef (negative control). As a result, mitochondrial D-loop primers can amplify the pig DNA, but not amplify the cow

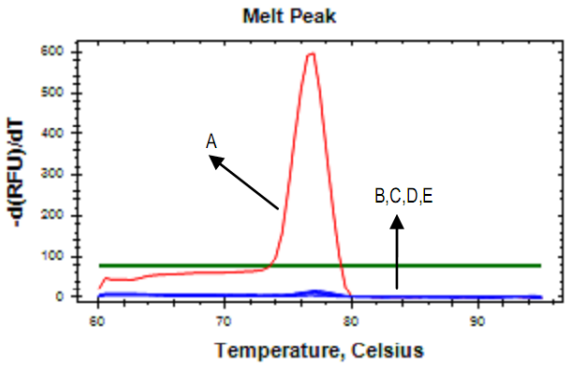


Figure 1. Melt peak of the specificity test of primers on pig DNA from fresh tissues compared with that on the DNAs of cow, chicken, goat and horse (A) pork DNA, (B) chicken DNA, (C) goat DNA, (D) horse DNA and (E) beef DNA

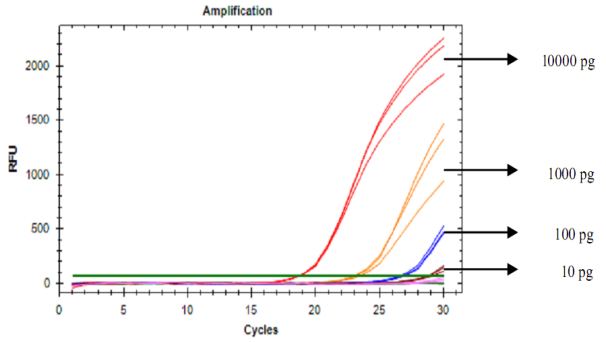


Figure 2. Curve amplification of the pig DNA from 100% shredded pork was used with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg

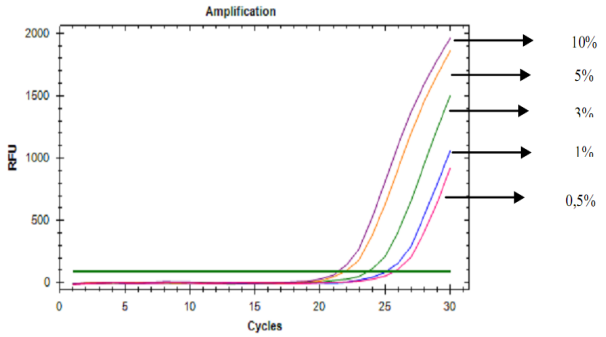


Figure 3. Curve amplification of pig DNA from the mixed shredded meat of pork-beef with the concentrations of 0.5; 1; 3; 5 and 10%

DNA. In the sample of the mixed shredded meat of pork: beef, the primers can also detect the pig DNA in the mixed cow DNA in all the concentrations. In the detection limit test, the pig DNA from 100% shredded pork was used with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg, so was the DNAs of the mixed shredded meat of pork:beef with the concentrations of 0.5; 1; 3; 5 and 10%. As a result, the mitochondrial D-loop22 primers can identify the pig DNA till 10 pg and the mixed shredded meat till 0.5% (Figure 2 and 3).

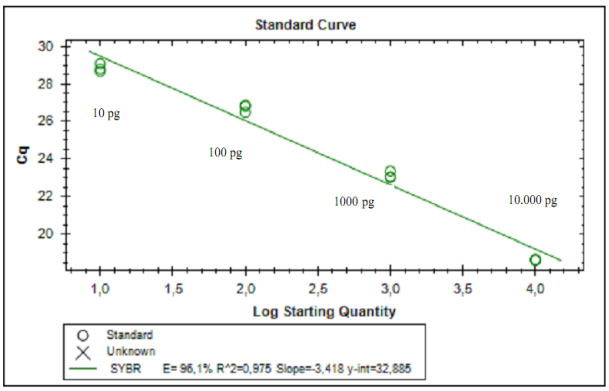


Figure 4. The standard curve of the results of DNA amplification in 100% shredded pork with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg showing that $R^2$ value was 0.975 and E value was 96.1%

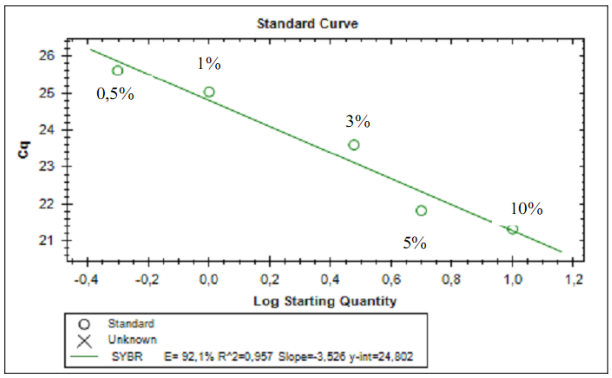


Figure 5. The standard curve of the results of DNA amplification in the mixed shredded meat of pork-beef with the concentrations of 0.5; 1; 3; 5 and 10% showing that $R^2$ value was 0.975 and E value was 96.1%

Figure 4 showed that the standard curve of the results of DNA amplification in 100% shredded pork with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg with mitochondrial D-loop22 primers, it can be showed that coefficient of determination ($R^2$) value was 0.975. The result did not meet the requirement because the acceptable value of the coefficient of determination is 0.980 (Bio-Rad , 2006). The efficiency value (E) obtained was 96.1%. The value met the requirement of good reaction with a range of E values from 90 to 105% (Bio-Rad, 2006). Figure 5 showed that standard curve of the results of DNA amplification in the mixed shredded meat of pork:beef with the concentrations of 0.5; 1; 3; 5 and 10% show that $R^2$ value was 0.957 and E value was 92,1%. The $R^2$ value obtained did not meet the requirement of 0.98, while the E value met the requirement.

The repeatability test was done to find out the repeatability of mitochondrial D-loop22 primers in identifying the pig DNA. The test was done using pig DNA from 100% shredded pork with the concentrations of 10000, 1000, 100, 10, 5 and 1 pg,

and the DNAs of the mixed shredded meat of pork-beef with the concentrations of 0.5; 1; 3; 5 and 10%. As a result, the average coefficient of variation (CV) for 100% shredded pork was 16.28% and for the mixed shredded meat of pork-beef was 7.46%. The analysis of pig DNA in the sample of shredded beef in market did not show the amplification, meaning that the shredded beef in market did not contain pork.

## Conclusion

The mitochondrial D-loop22 primers at the temperature of 59°C for primers attachment with the real time PCR method can specifically identify the target pig DNA among the DNAs of cow, chicken, goat, and horse, and the detection limit in shredded pork was till 10 pg and in the mixed shredded meat of pork-beef, it was till 0.5%, the average value of CV for 100% shredded pork was 16.28%, while for the mixed shredded meat of pork:beef, it was 7.46% (why in mix, its CV even less than 100% pork). The analysis of pig DNA in shredded beef in market did not show the amplification.

## Acknowledgements

Directorate General of Higher Education Ministry of National for funding the research through research project leading college SIM-LITABMAS (No: LPPM-UGM/346/LIT/2014). The leader of researcher is Prof. Dr. Sudjadi, MS., Apt.

## Refferences

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

Ali, M.E., Hashim, U., Mustafa, S., Che Man, Y.B., Dhahi, T.S., Kashif, M., Kamal Uddin, Md. and Abd Hamid, S.B. 2012. Analysis of pork adulteration in commercial meatballs targeting porcine-specific mitochondrial cytochrome b gene by TaqMan probe real-time polymerase chain reaction. Meat Science 91: 454–459.

Bio-Rad. 2006. Bio-Rad real time PCR application guide, Bio-Rad Laboratories: 2-10.

Che Man, Y.B., Aida, A.A., Raha, A.R. and Son, R. 2007. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control 18: 885–889.

Chen, F.C. and Hsieh, Y.H.P. 2000. Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. Journal of AOAC International 83: 79-85.

Chou, C., Lin, S., Lee, K., Hsu, C., Vickroy, T.W. and Zen, J. 2007. Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using cooper nanoparticle plated electrodes. Journal of Chromatoghrapy B 846: 230-239.

Dooley, J. J., Paine, K. E., Garrett, S. D. andBrown, H. M. 2004. Detection of meat species using TaqMan real-time PCR assays, Meat Science 68: 431–438.

Eugster, A., Ruf, J., Rentsch, J., Hubner, P. and Koppel, R. 2008. Quantification of beef and pork fraction in sausage by real-time PCR analysis : results of an interlaboratory trial. European Food Research Technology 227: 17–20.

Fatimah, S. 2013. Deteksi Cemaran Daging Babi dalam Campuran Bakso Ayam dengan real-time Polymerase Chain Reaction dan Spektrofotometeri Fourier Transform Infrared. Gadjah Mada University, Yogyakarta.

Fraga, D., Meulia, T. and Fenster, S. 2008. Real-Time PCR. Ohio.

Hernandes-Chaves, J.F., Gonzalez-Cordova, A.F., Rodriguez-Ramirez, R. and Vallejo-Cordoba, B. 2011. Development of a polymerase chain reaction and capillary gel elctrophoresis method for the detection of chicken or turkey meat in heat-treated pork meat mixtures, Analytica Chimica Acta 708: 149-154.

Kesmen, Z., Gulluce, A., Sahin, F., and Yetim, H. 2009. Identification of meat species by TaqMan-based real-time PCR assay. Meat Science 82: 444–449.

Kesmen, Z., Sahin, F. and Yetim, H. 2007. PCR assay for the identification of animal species in cooked sausages. Meat Science 77: 649–653.

Koppel, R., Ruf, J., and Rentsch, J. 2011. Multiplex real-time PCR for the detection and quantification of DNA from beef, pork, horse and sheep. European Food Research Technology 232: 151–155.

Martin, I., García, T., Fajardo, V., Rojas, M., Pegels, N., Hernández, P. E., Gonzalez, I. And Martin, R. 2009. SYBR-Green real-time PCR approach for the detection and quantification of pig DNA in feedstuffs. Meat Science 82: 252–259.

Meyer, R., Candrian, U., and Lüthy, J. 1994. Detection of pork in heated meat products by the polymerase chain reaction. Journal of AOAC International 77: 617–622.

Murugaiah, C., Noor, Z. M., Mastakim, M., Bilung, L. M., Selamat, J. and Son, R. 2009. Meat species identification and Halal authentication analysis using mitochondrial DNA. Meat Science 83: 57–61.

Nakyinsige, K., Man, Y. B. C. and Sazili, A. Q. 2012. Halal authenticity issues in meat and meat products. Meat Science 91: 207–214.

Nurjuliana, M., Che Man, Y. B., Mat Hashim, D. and Mohamed, A. K. S. 2011. Rapid identification of pork for halal authentication using the electronic nose and gas chromatography mass spectrometer with headspace analyzer. Meat Science 88: 638–644.

Rohman, A., Sismindari, Erwanto, Y., and Che Man, Y. B. 2011. Analysis of pork adulteration in beef meatball using Fourier transform infrared (FTIR) spectroscopy. Meat Science 88, 91–95.

Sahilah, A.M., Norhayati, Y., Norrakiah, A.S., Aminah, A. and Wan Aida, W. M. 2011. Halal authentification of raw meats using PCR amplification of mitochondrial DNA. International Food Research Journal 18: 1489-1491.

Sambrook, J., Fritsch, E. F., dan Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory Press.

Soares, S., Amaral, J. S., Oliveira, M. B. P. P. and Mafra, I. 2013. A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. Meat Science 94: 115–120.

Soares, S., Amaral, J.S., Mafra, I. and Oliveira, M.B.P.P. 2010. quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. Meat science 85: 531-536.

Syahruni, R., Sismindari and Astuti. 2013. Analisis babi pada burger menggunakan real time PCR. Gadjah Mada University, Yogyakarta.

Tjondro, F. and Sismindari. 2012. Detection of pork in burger using real-time PCR with LEF primer. Proceeding of the 2nd international seminar on halalness and safety food and pharmaceutical product 17-18 Oct 2012. Yogyakarta.

Xu, L., Cai, C. B., Cui, H. F., Ye, Z. H. and Yu, X. P. 2012. Rapid discrimination of pork in halal and non-halal chinese ham sausages by fourier transform infrared (FTIR) spectroscopy and chemometrics. Meat Science 92: 506–510.