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A taq-man multiplex real-time pcr for identification of porcine and canine dna in halal food

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ABSTRACT

The efficient and timely detection of prohibited animals in Halal food is very important. This study aimed to establish a rapid detection method for porcine and canine DNA in a food product. Porcine and Canine DNA were Contaminated in halal food products using multiplex Real-Time PCR and showed positive specificity only in Porcine and Canine meat but showed negative results in chicken, cow, and fish. The results from porcine showed that the lowest level is at 0.001% and 0.1% in raw meat and cooked meat heated at 120 degrees celsius for 15 min, and 0.1% in diluted canine DNA respectively. The method for detection of the contamination of porcine and canine DNA in Halal food products with multiplex real-time PCR technique is suitable and high sensitivity for detecting the DNA of prohibited animals in meat products and raw materials, such as detecting contaminated porcine DNA in sweet jelly and soup cubes.

Keywords: Multiplex Real-time PCR, Halal food, Canine DNA, porcine DNA

INTRODUCTION

The identification of species in food is a very important issue concerning food composition due to economic, health, and religious issues (Mahsa *et al*., 2018). In most countries, pork and lard are serious matters because of some religions such as Islam and Judaism, foods containing pork and canine sources are Haram (unlawful or prohibited) for Muslims to consume. The past ago has seen patterns of protein in species identification animal species such as enzyme-linked immunoadsorbent assay (Wissiack *et al*, 2003), these methods have been reported to have limitations in use and problems in specificity and high cost.

A major problem in protein-based analysis is that proteins are easily denatured during heat treatment (King and Kurth, 1982). Therefore, a very important reason protein base analysis has been replaced by the DNA base method (Saiki *et al*, 1988; Ali et al, 2006; Lahiff *et al*, 2001). DNA-based methods have been well received because of the stability of DNA under the heat process and its efficient amplification by PCR [Jerilyn *et al*, 2003; Matsunaga *et al*, 1998, Maharat *et al*, 2005], Real-time PCR (Laube *et al*, 2003; Miguel *et al*, 2005). However, real-time PCR technology is still expensive if needed to examine more than one species of animals. Therefore, multiplex real-time PCR techniques were used to detect the contamination of two kinds of prohibited animals. Multiplex Real-time PCR helped decrease analysis time and cost. These advantages, a Multiplex real-time PCR method for the identification of pork and canine in meat products. This study showed that primer and probe for the detection of the contamination of porcine/Canine DNA in meat products and production raw materials by comparing the sensitivity of primer and probe in the repetitive elements and mitochondrial DNA apply to authentication of meat products.

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MATERIALS AND METHOD

Sample selection

Raw meat and heated meat mixtures

Pork meat and chicken meat were acquired from a supermarket. The raw meat and heated meat mixtures were acquired sample meat mixed in different ratios such as 1 %, 0.1%, 0.01%, 0.001%, 0.0001%, and 0.00001%, The mixture meat was stored at -20 °C until analyzed.

Diluted canine DNA

Diluted canine DNA at different intensities such as 1 %, 0.1%, 0.01%, 0.001%, and 0.0001%, diluted Canine DNA was stored at -20 ºC until analyzed.

Halal food product

A total of 24 commercial meat products were purchased from local markets. All commercial meat samples were stored at -20 ºC until analyzed.

Porcine DNA, Canine DNA were positive control and chicken DNA was a negative control

DNA extraction

DNA was extracted using the Genomic DNA Mini Kit (Tissue) (Takara, Nojihigashi, Japan). Usually, 5 mg of each sample material was lysed with 20 µL of Proteinase K and 180 µL of Lysis buffer solution in a heating block at 60 °C for half an hour. Then, after lysis, add 180 µL lysis buffer and incubate them at 70 °C for 10 minutes. The mixture was eluted with 200 µL of elution buffer. The concentration was determined by spectrophotometer and adjusted by dilution to 20 ng/L.

Primer and Probe Design

Real-time PCR primer and probe for detected canine DNA, the chromosome X gene of canine (GenBank accession no: AC188657) primer were canine F: CGA GGT GCC TAT GAG GTG AAC, Canine-R: TCC CTT TTT CAA TAG GCC CTA CTG to amplify a 98 bp fragment. The canine-specific probe designed in this study with the primer 3.0 program was CGA GGT GCC TAT GAG GTG AAC. A hexachloro-6-carboxyfluorescein (HEX) was attached to 5′ ends of the probe. The quencher was 6-carboxy-tetra methylrhodamine (TAMRA), which was added to the 3′ ends of the probe. porcine DNA, the repetitive element gene (GenBank accession no: AF392878) primer were pigpre: GGA TCC GGC ATT GCC GTT AG, pigpre-R: GTC TTT TTT TGC CAT TTC TTG G to amplify 116 bp (Jerilyn, 2004). The porcine-specific probe designed in this study with the primer 3.0 was CTAGCCTGGGAACCTCCATA. A fluorescent dye, 6-carboxyfluorescein (FAM) was attached to the 5′ ends of the probe. The quencher was 6-carboxy-tetramethylrhodamine (TAMRA), which was added to the 3′ ends of the probe.

Multiplex Real-time PCR protocol

Mix PCR 20 µl, Probe, and primer at a concentration of 200 nM. Real-time PCR was performed on ABI 7300 real-time system (ABI, Germany) step-cycle program: pre-cycle of 50 °C for 2 min and pre-denaturation of 95 °C for 10 min for completely denature the DNA template, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing and extension at 60 °C for 60 sec. Examination of results in the appearance of the Fluorescence signal in graph Amplification plot and Ct number which can examine the Fluorescence signal in the positive sample. For the negative sample, provided only the background fluorescence in the graph Amplification plot.

RESULT AND DISCUSSION

Specificity test

The specificity of a multiplex real-time PCR assay for two species: was tested by analysis of DNA from different animal species. The porcine and canine-specific system amplified fragments from pork DNA. whereas no amplification was obtained from other species, Chicken, Cow, and Fish (Figure.1) within 40 cycles of amplification. The result is shown in picture 1. the assay was specific for the porcine repetitive element gene and canine Cyt b gene that primers and probes specific to the meat

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showed no cross-reaction with any of the non-target meat. The results are consistent with research by Calvo *et al*., which detected porcine DNA in meat (Calvo *et al*, 2001)**.** The limits of detection in raw meat samples (pork mixed with other meat) and mixed heated meat of 120 ºC in different ratios (Figure 2) The lowest level is at 0.001% and 0.1% in raw meat and heated meat of 120 ºC respectively in Figure A, B. We also examined the sensitivity of diluted canine DNA at ratios of 1%, 0.1%, 0.01%, 0.001%, and 0.0001% It was found that the detection limits in diluted canine DNA gave the best results at a ratio of 0.1%. (Figure 3). The Multiplex Real-time PCR reaction is a highly sensitive method. The lowest level is at 0.001% and 0.1% in raw meat and heated meat at 120 ºC respectively. This corresponds with the research by Ali, 2006 After the heated meat pieces could not be measured (Ali *et al.*, 2003) This was also seen in the sauce of the baked product, PCR detection may fail. This could be the result of the charring of meat as well as the denaturation of the DNA. We also examined the sensitivity of diluted Canine DNA at ratios of 1%, 0.1%, 0.01%, 0.001%, and 0.0001%. It was found that the detection limits in diluted Canine DNA gave the best results at a ratio of 0.1%. A Taq Man Multiplex Real-time PCR the for the detection of contamination of porcine and canine DNA in halal food such as gelatin and animal fat, it was found that porcine DNA showed which can detect Fluorescence signals as shown in table I. In food products and raw materials such as gelatin and animal fat, it was found that porcine DNA was detected in a sample. In some processed food from fish was positive DNA in both animals. For the samples which are chicken sausage (no Halal mark), fish sausage (no Halal mark), and pork sausage, the contamination of porcine DNA can detect Fluorescence signals. It was found that the Desserts sample mixed with gelatin gives negative results except for sweet jelly number 1, shown which can detect Fluorescence signals. This corresponds with the research result by Rene Koppel and his colleague which found out that the detection of porcine DNA contamination in the sample undergoing high heat by Multiplex Real-time PCR technique can examine the least quantity at 0.1% in the sample containing pork in little amount and mixed with other substances such as soup cubes or gelatin from pork (Rene et al., 2008). Results and discussion should be combined in the manuscript. It is should be described concisely. Text, tables, and figures must be internally consistent. Discussion should involve the significant findings presented with relevant and extensive discussion.

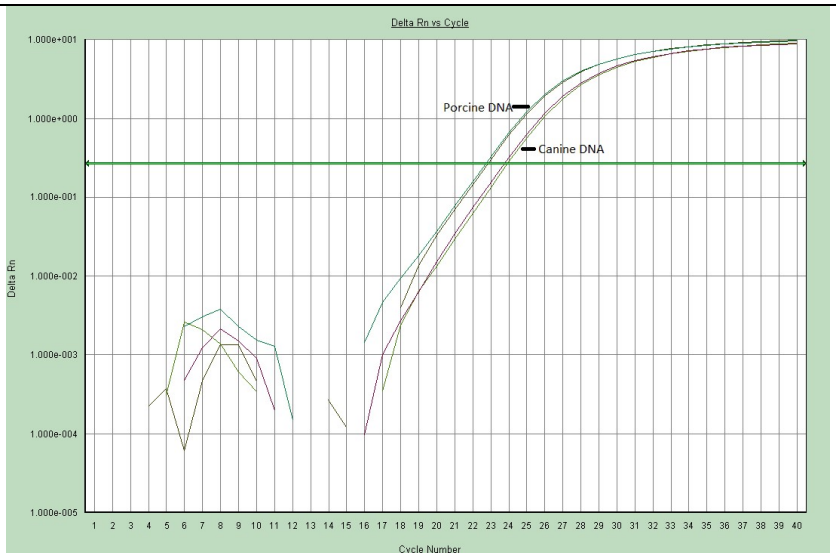

Figure 1. The amplification plot picture demonstrates the result of increasing the quantity of porcine and canine DNA by the Multiplex Real-time PCR technique. In the appearance of Fluorescence signal in the graph Amplification plot for fish, beef, and chicken meat

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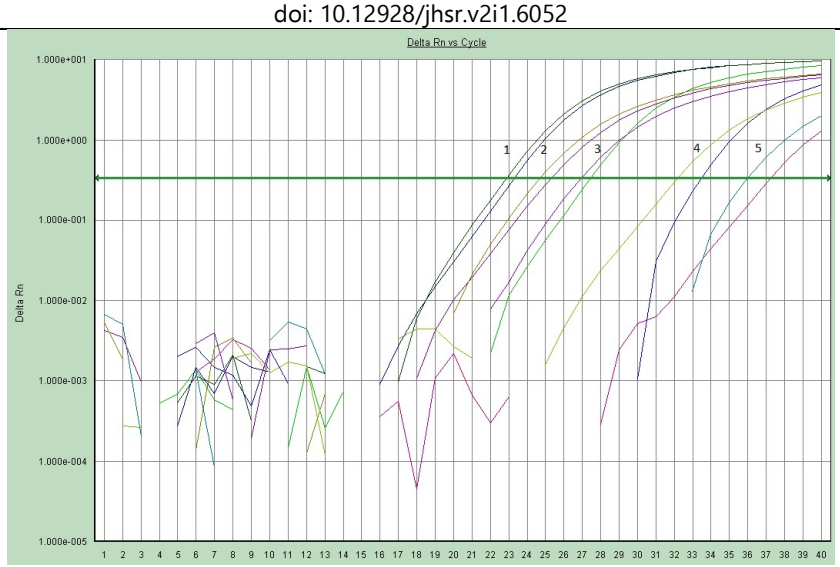
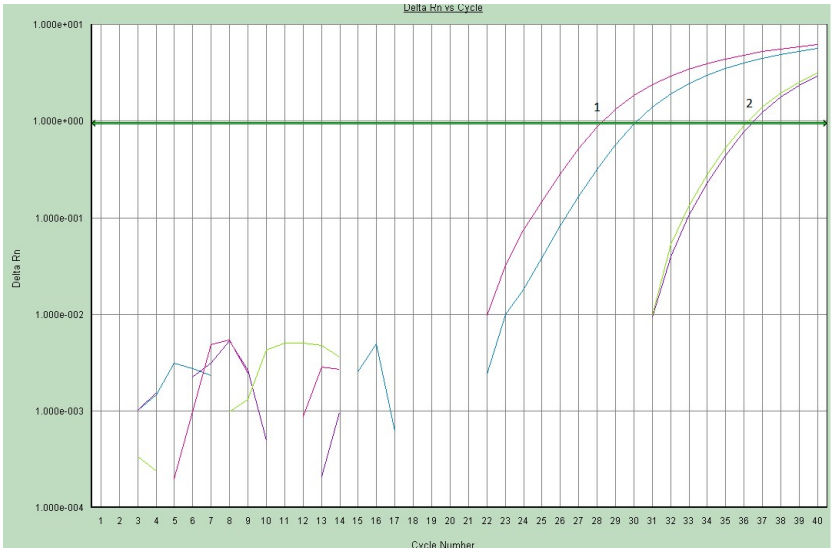


(A)



(B)

Figure 2. Illustrating Amplification Plot picture, showing Fluorescence signal detectable in raw meat (pork mixed with other meat) in different ratios of 2=1 %, 3=0.1%, 4=0.01%, 5=0.001%, 6=0.0001%, 1= positive controller is porcine DNA in Figure A. The negative controller is chicken meat. The mixed meat was heated at 120 °C in different ratios of 1=1 %, and 2=0.1%, the positive controller is porcine DNA in Figure B.

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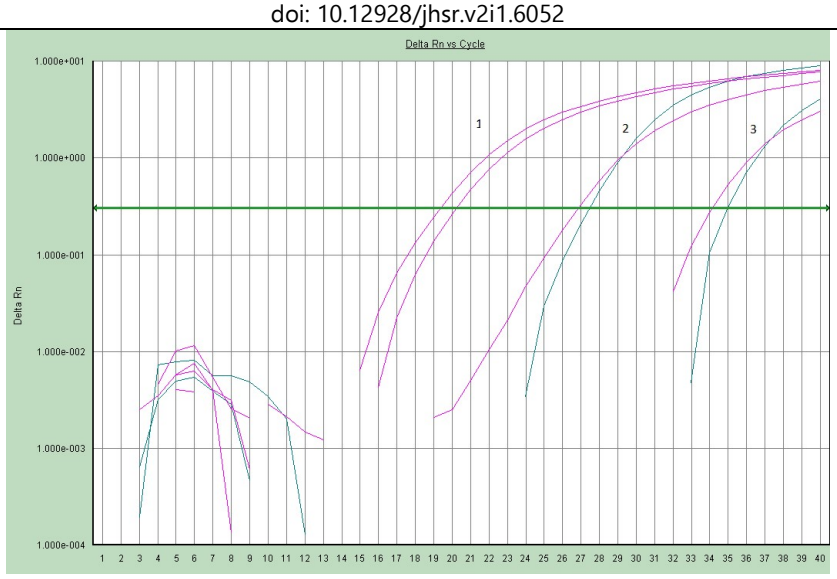

Figure 3. Amplification Plot picture, showing detectable diluted canine DNA in different ratios such as 1=1 %, 2=0.1%, 3=0.01%. The positive controller is fresh pork, the negative controller is chicken meat.

Table I. Summarizes the result of porcine and canine DNA detection with the real-time PCR technique in Halal food sample

Sample	Porcine DNA	Canine DNA
1. Fried Fish Chip	-	-
2. Bird's nest 1	-	-
3. Mini Cocktail Chicken	-	-
4. Chicken Thai Sausage	-	-
5. TOFU Fish egg	-	-
6. Filament fish Stick	-	-
7. Flavoured Fish Ball	+	+
8. Tufted SEAFOOD Roll	-	-
9. Fish finger	-	-
10. Fried seafood TOFU CURD	-	-
11. Tofu sandwich	-	-
12. Salmal ball	-	-
13. Bird's nest 2	-	-
14. Sai Our	+	-
15. Sweet jelly 1	+	-
16. Sweet jelly 2	-	-
17. Sweet jelly 3	-	-
18. Sweet jelly 4	-	-
19. Jelly rainbow	-	-
20. Soup cube1	+	-
21. Soup cube 2	+	-
22. Soup cube 3	+	-
23. Sweet jelly 5	-	-
24. Sweet jelly 6	+	-

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CONCLUSION

Based on the result of this study, the primer and probe used have specificity and high sensitivity. This corresponds with the research by Rahman and his colleagues which can detect the contamination of porcine DNA by using the multiplex Real-time PCR technique (Rahman *et al*, 2016). In food products and raw materials such as gelatin and animal fat, it was found that we can detect porcine DNA contamination in the sample containing a little amount of pork undergoing another production process such as soup cubes, gelatin from pork, or samples that are sweets mixed with gelatin. the result indicates that determination by PCR is affected by the high temperature and size of the DNA fragment to be amplified (Mane *et al*, 2013). For this reason, Useful DNA is small in size to amplify. However high temperatures could break down the DNA. The sample contains porcine DNA in little amounts and is mixed with other substances or samples which are sweets mixed with gelatin which can be detected or given positive results by detecting the Fluorescence signal in the sample giving a positive.

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