Original paper

Fast and Sensitive Real-time PCR-based Detection of Porcine DNA in Food Samples by Using EvaGreen Dye

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Accidental and/or incidental adulterations of foods by porcine ingredients are common in the globalized food processing industry. Food mislabelling and fraudulent substitutions of non-porcine ingredients with porcine ones are objectionable to those who abstain from porcine derived products due to habitual (e.g., vegans and vegetarians), medical (e.g., porcine allergies), legal (fraudulent labelling), economic (e.g., substitution of expensive meat with cheaper pork meat) and cultural or religious grounds (e.g., Islamic and Jewish dietary restrictions). Thus, a strong demand exists for a fast and sensitive method for quantitative sensing of porcine DNA in food. In this study, we are reporting the development of probe-free real-time PCR assay with new primer sets targeting the *cytochrome b* gene for the fast and sensitive detection of porcine DNA in real food samples. Standard curve was developed with six ten-fold dilutions of the DNA standard and the assay successfully detected up to 0.00001 ng/ μ L of porcine DNA and as low as 0.001 % porcine adulteration in raw pork-chicken binary mixture. The standard curve indicated a linear regression of R^2 value of 0.990 and an efficiency of 92.5 %. The Ct value range for the detected pork DNA from the 35 food samples tested was 16.03–28.76. We confirmed the assay's specificity to porcine DNA against nine non-porcine animal species and 6 vegetables species.

Keywords: real-time PCR, porcine DNA detection, porcine adulteration, food analysis

Introduction

Food need to conform to a variety of criteria in order to address the differences in consumers' tastes, health conditions, legal regimes and cultural orientations including religious obligations (Tieman and Hassan, 2015; Fang and Zhang, 2016; Fischer, 2016; Lubis *et al.*, 2016). Since the EU meat-scandal in 2008, fraudulent adulterations of food products have become a grave concern. Instances of adulteration may be intentional, like substituting expensive meat with cheaper pork meat for economic gain; or unintentional, including cross-contamination during co-processing or mislabelling at the processing plants. Pork adulteration in food labelled as pork-free is one of the big concerns for people of certain beliefs and cultures (Tieman and

Hassan, 2015; Lubis *et al.*, 2016).

There are existing and emerging regulatory frameworks to address the issues which necessitate the food manufacturers to maintain transparency in food labelling to ensure the traceability of ingredients (Fortin, 2016). In reality, mislabelling is still a major concern especially for meat products which are susceptible to adulterations due to co-processing of meats of different species at the same processing facilities (Ballin, 2010). Despite descriptive labelling, past cases of fraud by dishonest manufacturers stirred inevitable doubts. For example, porcine DNA has been detected in a food product labelled as pork-free (Demirhan *et al.*, 2012). Evidently, the situation arrested the demand for analytical

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methods to detect such adulteration and accordingly methods to detect and quantify the presence of porcine meat in meat products have been developed. Nevertheless, developing a more reliable meat species detection protocol is crucial to help food industries maintaining compliance with legal, ethical, and medical concerns so that the consumers may avoid food which are unsuitable for them.

Immunological (Asensio *et al.*, 2008), protein- and DNA-based electrophoretic, and chromatographic (Toorop *et al.*, 1997) techniques have been employed in species identification. However, protein-based methods have limited applicability for species detection in cooked, baked, or heat-treated food products due to protein denaturation at high temperature (Cai *et al.*, 2012; Nakyinsige *et al.*, 2012). In contrast, due to the relative stability of DNA under heat and pressure treatments (Soares *et al.*, 2013; Karabasanavar *et al.*, 2014) – it is widely used in polymerase chain reaction (PCR) for fast, specific, and sensitive detection of meat species (Cammà and Domenico, 2012; Soares *et al.*, 2013; Fang and Zhang, 2016). Quantitative real-time PCR (qPCR) is widely used as a powerful platform for simultaneous amplification and quantification of nucleic acids (Mao *et al.*, 2007). Unlike end-point PCR, qPCR advantageously monitor the amplification after each PCR cycle by detecting the changes in the fluorescence intensity from the fluorescent molecules. A cheaper alternative to the widely use fluorogenic probe in most qPCR analysis is to use DNA-binding dye as the second fluorogenic agent to detect qPCR fluorescence (Soares *et al.*, 2013). DNA-binding dye increases fluorescence significantly, either by intercalating into the double-stranded DNA or by binding to the minor grooves of the double-stranded DNA.

Dye-based qPCR assays have been reported in many studies to detect animal DNA in meat mixtures. Fajardo *et al.* (2008) used SYBR Green I dye to detect and quantify different species of deer DNAs. Soares *et al.* (2013) detected and quantified porcine and poultry DNA by using the same type of DNA-binding dye. Detection of animal meat species such as porcine (Amaral *et al.*, 2017), equine (Meira *et al.*, 2017) and hare (Santos *et al.*, 2012) by using EvaGreen dye qPCR were previously reported.

An example of a commonly used DNA-intercalating dye is SYBR Green I DNA-binding dye that enhances fluorescence by more than 10,000-fold upon binding to the minor groove of the double-stranded DNA (Dragan *et al.*, 2012). However, SYBR Green I dye may inhibit PCR at high concentrations by causing mis-priming (Karsai *et al.*, 2002). In contrast, at low concentrations, SYBR Green I dye may compromise the strength of fluorescence signal that renders DNA melting curve data unreliable (Varga and James, 2006). Also, SYBR Green I dye tends to bind to long, G-C rich PCR products which make it unsuitable for multiplexed PCR analysis (Giglio *et al.*, 2003). Nevertheless, SYBR Green I dye has been popular in qPCR analysis for animal species identification due to its commercial

availability (Fajardo *et al.*, 2008; Soares *et al.*, 2013).

On the other hand, EvaGreen dye is a third generation DNA-binding dye developed by Biotium Inc (Hayward, CA, USA) which is an alternative to SYBR Green I dye (Meira *et al.*, 2017). The maximum absorption and emission wavelengths of the pure EvaGreen dye are 495 nm and 525 nm, respectively which are shifted to 500 nm and 530 nm in the presence of DNA (Mao *et al.*, 2007). Unlike SYBR Green I, EvaGreen was developed with enhanced fluorescence to offer greater signals, enhanced sensitivity and improved stability without impairing PCR reactions even at higher concentrations (Wang *et al.*, 2006). Real-time PCR with EvaGreen dye has also been used to detect porcine (Amaral *et al.*, 2017), equine (Meira *et al.*, 2017) and hare (Santos *et al.*, 2012) meats.

For this research, EvaGreen DNA-binding dye was used in the qPCR assay to detect and quantify porcine DNA in food samples since EvaGreen is relatively cheaper and can be used at higher concentrations without compromising the signals and sensitivity (Wang *et al.*, 2006). This study utilized a novel primer sequences to detect the *cytochrome b* gene in porcine species.

Materials and Methods

2.1 Materials

2.1.1 Food samples A total of 35 samples consisting of 7 raw and 28 processed foods from both local and international suppliers were purchased from local supermarkets. 5 of the raw samples were artificially contaminated binary mixtures of raw porcine and raw chicken meats with varying weight percentages (10 % to 0.001 %) of porcine meat. Commercial porcine genomic DNA (Zyagen) was used as the positive porcine DNA control and eight ten-fold serial dilutions of this DNA were prepared for determining the assay's sensitivity.

Genomic DNA of a variety of non-porcine animal species and raw vegetable ingredients commonly found in processed foods was included as a test for the assay's specificity. Those samples were bovine, buffalo, chicken, duck, goat, horse, ostrich, sheep, turkey, wild boar, yellow onion, garlic, green chilli, black pepper, soybean and potato.

2.2 DNA extraction DNA was extracted from approximately 200 mg of each of the homogenised food samples and the non-porcine animal species samples according to the user manual of the NucleoSpin® Food kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Briefly, the homogenised sample was incubated with Proteinase K and lysis buffer for 30 minutes at 65 °C followed by centrifugation to remove the cell debris. The supernatant was loaded into a spin column after mixing with binding buffer and ethanol. The column was washed three times with wash buffers after the DNA solution was bound to the column and finally, the purified DNA was eluted from the column by elution buffer.

The extraction of vegetable's DNA from approximately 200 mg of each homogenised vegetable samples according to

the instruction manual of DNeasy® mericon® Food kit (QIAGEN, Hilden, Germany) with few modifications in incubation and centrifugation time. In brief, Proteinase K and food lysis buffer were added to the homogenised sample and incubated for 3 hours at 60 °C. Cell debris was separated by centrifugation and the clear supernatant was added to a chloroform solution follow by another centrifugation. Then, the upper aqueous phase was pipetted out, mixed with binding buffer and loaded into a spin column. The column was washed once with wash buffers followed by a series of centrifugation to discard the flow through. Subsequently, elution buffer is loaded into the spin column in order to obtain the purified DNA.

The concentration and purity of the extracted and purified DNA were measured by spectrophotometric method by using NanoPhotometer™ P-Class (Implen, Munchen, Germany). The DNA concentration was read as the absorbance peak at 260 nm while its purity was determined from the optical density of A260/A280 ratio. The extracted DNA was stored at –20 °C until real-time PCR assay.

2.3 Primers design The species-specific primers and probes were designed to target a fragment of porcine mitochondrial *cytochrome b* gene (accession number AM492586.1). Its sequence was retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov>). The primers (Table 1) were designed by using PrimerQuest Tool, at IDT (Integrated DNA Technologies, IDT PTE, Singapore) website (<http://www.idtdna.com/Primerquest/Home/Index>). The designed primers match the region of 468 bp to 570 bp of the porcine mitochondrial genome where this sequence is part of the *cytochrome b* gene. The specificity of the designed primers and probe were analysed in silico by using the BLAST tool of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to prevent non-specific binding of the primers to DNA of species other than porcine by ensuring that the sequences were specific only to porcine and not to any other species. Other than matching the porcine *cytochrome b* gene sequence, these primers were chosen based on its 100 % alignment with the mitochondrial genome sequence of varieties of porcine (*Sus scrofa*) species upon *in-silico* specificity check with BLAST. IDT supplied the primers.

2.4 Real-Time PCR assay The qPCR reaction volume was 20 µL, with 19 µL qPCR reagents mixture and 1 µL DNA template. The qPCR reagents mixture contained 1× PCR buffer II, 0.2 µM dNTP mix, 0.15 µM of each of the forward and the reverse primers, 1× EvaGreen dye, 0.1× ROX as the passive reference, 0.625 U AmpliTaq DNA polymerase, and 2.5 mM MgCl₂. All qPCR assays were run with a known positive control - the commercial porcine genomic DNA as template and nuclease-free water as a known no-template control (NTC). The qPCR assay was performed in the 7500 Fast Real-Time PCR System (ABI) with the fast thermal cycling profile at 95 °C for 20 seconds followed by 40 cycles at 95 °C for 3

Table 1. Oligonucleotide primers for dye-based real-time PCR assay

Designation	Sequence (5' – 3')
Forward Primer	CGGAACAGACCTCGTAGAATG
Reverse Primer	GGTAATGATGAATGGCAGGATAAAG

seconds and 60 °C for 30 seconds. A melt curve analysis was done on the positive DNA (10 ng/µL Pork DNA). The 7500 Software version 2.3 was used for data collection and processing.

2.5 Specificity efficiency, and sensitivity test The specificity of the dye-based qPCR assay using porcine-specific primers was tested against genomic DNAs from 9 non-porcine species (bovine, buffalo, chicken, duck, goat, horse, ostrich, sheep, turkey) 1 porcine species (wild boar) and 6 vegetables species (onion, garlic, chilli, black pepper, soybean and potato). Sensitivity of the assay was measured by eight ten-fold serial dilutions of porcine genomic DNA ranging from 10 to 0.00001 ng/µL. The standard curve was generated by plotting the Ct values against the log of the DNA concentrations.

2.6 Real food samples analysis The dye-based qPCR assay was used to detect porcine DNA in all 35 food samples comprised of pork meat, pork-free meat, and binary mixtures pork-chicken raw meat.

Results and Discussion

3.1 Development of assay and analysis of extracted DNA In this research, an EvaGreen dye-based qPCR was developed using previously unreported primers for porcine DNA detection for food sample analyses. EvaGreen dye was chosen to improve assay's sensitivity as it does not inhibit PCR reaction even at high concentrations (Wang *et al.*, 2006). The concentration range of the DNA extracted from 35 food samples was 10 to 664 to ng/µL while the purity ranged from 1.42 to 2.02. Some food samples showed low DNA concentrations. Overall, the DNA purity was high, indicating the high quality of the purified DNA (Ali *et al.*, 2015). This has also ascertained the acceptability of the commercial DNA extraction kit used to extract DNA from sample meat products and the raw meat mixtures used in this research.

3.2 Specificity The specificity of primer pairs (Table 1) used for this EvaGreen dye assay for specific amplification of porcine DNA was confirmed by testing the assay against DNA from 9 non-porcine, 1 porcine animal species and 6 vegetable species (Table 2). Absence of amplifications for any of the vegetable species and the non-porcine species (Supplementary 1) except for wild boar (Table 2) indicated that the primers' target sequence also occur in wild boar's *cytochrome b* gene. This indicated versatility of the designed primers as wild boar meat can also be used as an alternative to porcine meat. Therefore, the assay developed was highly specific to porcine species. The melting temperatures (T_m) of the wide range

Table 2. Results of the specificity test of the dye-based real-time PCR

Species DNA ^a	Mean Ct value ^b	SD ^c	Number of positive replicates
Porcine	15.11	2.1E-2	3/3
Bovine	nd ^d	nd	0/3
Buffalo	nd	nd	0/3
Chicken	nd	nd	0/3
Duck	nd	nd	0/3
Goat	nd	nd	0/3
Horse	nd	nd	0/3
Ostrich	nd	nd	0/3
Sheep	nd	nd	0/3
Turkey	nd	nd	0/3
Wild Boar	16.98	2.3E-2	3/3

^aThe DNA of different species were all made to have equal concentration.^bThe Ct values are the mean of replicate assays (n = 3).^cSD – standard deviation.^dnd – porcine DNA not detected. **Fig. 1.** Standard curve of Table 1 EvaGreen assay (A) Melting curve analysis and (B) The amplification plot constructed from 10-fold dilution of known concentrations of porcine DNA. The melting curve showed the T_m of detectable Porcine DNA was 83.08 °C while no peak was detected on the NTC. The amplification plot showed the highest concentration of diluted porcine DNA templates (10 ng/μL) was amplified at earlier cycle as compare to lower concentrations which were amplified at later cycles.

concentrations of porcine DNA were determined through the melting curve analysis after the amplification cycles and were found to be at 83.08 °C (Figure 1-A). The specificity of the assay was further confirmed with gel electrophoresis that showed high specificity of the primers to the target DNA, producing only single positive band for porcine (positive control) and wild boar, and none for the fifteen non-target species analysed (Supplementary 1).

3.3 Sensitivity The sensitivity of the assay was determined

by testing eight ten-fold serially diluted porcine DNA templates viz., 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 ng/μL (Figure 1B). Amplifications were observed for all DNA template concentrations except for the lowest one. Therefore, the lowest detectable porcine DNA concentration was 0.00001 ng/μL (Figure 1B). The limit of detection (LOD) of the assay was comparable to that obtained in other EvaGreen dye-based study (Amaral *et al.*, 2017) while it was lower than that obtained by using SYBR Green I dye (Soares *et al.*, 2013).

Supplementary 1. Gel electrophoresis result of the specificity analysis. (1) NTC; (2) 50-bp DNA Ladder; (3) Positive control (porcine); (4) Wild boar; (5) Bovine; (6) Buffalo; (7) Chicken; (8) Duck; (9) Goat; (10) Horse; (11) Ostrich; (12) Sheep; (13) Turkey; (14) Onion; (15) Garlic; (16) Chili; (17) Black pepper; (18) Soybean; (19) Potato.

Fig. 2. Standard curve of porcine DNA. The C_t values were plotted against Log of six ten-folds DNA dilutions (10, 1, 0.1, 0.01, 0.001, 0.0001 ng/ μ L). The R^2 value obtained was 0.990 and the efficiency was noted to be 92.5%. Standard curve constructed from mean values of C_t values of replicate assays ($n = 3$).

This EvaGreen dye-based qPCR assay showed better sensitivity compared to the other probe-based qPCR studies (Cai *et al.*, 2012; Cammà and Domenico, 2012; Yusop *et al.*, 2012; Iwobi *et al.*, 2015; Kim *et al.*, 2016).

3.4 Efficiency The first six concentrations (10 to 0.0001 ng/ μ L) that showed positive amplifications (Figure 1B) were used to construct the standard curve that plotted C_t value against the logarithm of DNA concentrations (Figure 2). The standard curve showed very good linear regression ($R^2 = 0.990$) with a slope of -3.517 . The efficiency of the assay was 92.5%

Fig. 3. Amplification plot of Porcine DNA from pork food sample ($C_t = 16.667$) compared to the positive control ($C_t = 11.804$). Porcine DNA of 10 ng/ μ L acted as the positive control of the reaction and was amplified at earlier cycle. This was due to the high concentration of porcine DNA in the positive control as compare to that in the food sample tested

as calculated from the slope of the standard curve by using the formula $E (\%) = (10^{-1/\text{slope}} - 1) \times 100$ (Cammà and Domenico, 2012; Druml *et al.*, 2015) which indicated the robustness and precision of the newly developed assay (Bustin *et al.*, 2009). Accordingly, we attested the dynamic quantification range for this qPCR assay was between 10 ng/ μ L and 0.0001 ng/ μ L.

3.5 Validity and applicability The applicability of this new dye-based qPCR assay was validated by applying it to detect porcine DNA in 35 food samples. Figure 3 shows the amplification curves of porcine DNA from one of the pork food

Table 3. Results of dye-based real-time PCR assay for food samples

Sample no.	Sample type ^a	Mean Ct value ^b	SD ^c	Number of positive replicates	Presence of porcine DNA ^d
1	Chopped pork and ham	16.62	4.4E-2	3/3	+
2	Spiced pork cubes	16.14	7.2E-2	3/3	+
3	Cocktail skinless sausages	17.07	1.0E-1	3/3	+
4	Pork luncheon meat	16.27	2.9E-2	3/3	+
5	Pork mince with bean paste	16.45	3.8E-2	3/3	+
6	Pork luncheon meat with black pepper	18.05	7.1E-2	3/3	+
7	Pork and bamboo shoot	23.10	3.4E-1	3/3	+
8	Sliced ham	21.74	6.0E-2	3/3	+
9	Pork short sausages	17.48	7.8E-3	3/3	+
10	Boar meat	16.03	6.5E-2	3/3	+
11	Marshmallow	nd ^e	nd	0/3	-
12	Marshmallow	nd	nd	0/3	-
13	Marshmallow	nd	nd	0/3	-
14	Beef meat loaf	nd	nd	0/3	-
15	Corned beef	nd	nd	0/3	-
16	Chicken frankfurters	nd	nd	0/3	-
17	Chicken luncheon meat	nd	nd	0/3	-
18	Corned beef	nd	nd	0/3	-
19	Chicken luncheon meat	nd	nd	0/3	-
20	Mutton luncheon with chicken	nd	nd	0/3	-
21	Corned beef	nd	nd	0/3	-
22	Beef loaf	nd	nd	0/3	-
23	Corned mutton	nd	nd	0/3	-
24	Chicken luncheon meat	nd	nd	0/3	-
25	Beef curry	nd	nd	0/3	-
26	Chicken luncheon meat	nd	nd	0/3	-
27	Corned ostrich	nd	nd	0/3	-
28	Lamb curry with potatoes	nd	nd	0/3	-
29	Duck meat	nd	nd	0/3	-
30	Beef luncheon meat	nd	nd	0/3	-
31	Pork-beef raw meat mixture (10 % pork, w/w)	19.73	1.6E-2	3/3	+
32	Pork-beef raw meat mixture (1 % pork, w/w)	23.34	4.2E-2	3/3	+
33	Pork-beef raw meat mixture (0.1 % pork, w/w)	27.11	1.6E-1	3/3	+
34	Pork-beef raw meat mixture (0.01 % pork, w/w)	28.30	5.6E-2	3/3	+
35	Pork-beef raw meat mixture (0.001 % pork, w/w)	28.76	7.9E-2	3/3	+

^aAll the food samples were combinations of raw and processed foods supplied by few countries, bought from local supermarkets. Similar name samples were of different brands.

^bThe Ct values are the mean of replicate assays (n = 3).

^cSD – standard deviation.

^dResult of real-time PCR: +, positive for porcine DNA; -, negative for porcine DNA.

^end – porcine DNA not detected.

sample compared to the 10 ng/μL porcine DNA (positive control). This illustrates the capability of the assay in amplifying food sample containing porcine DNA. The assay successfully detected porcine DNA in 15 out of the 35 samples tested (Table 3), where all 15 of the positive samples contained porcine or boar DNA. This indicates the validity of this dye-

based qPCR assay in successfully detecting the pork DNA in the food samples.

The standard curve was developed for the determination of the efficiency of the assay and was used to quantify porcine mitochondrial DNA from five pork-chicken raw meat binary mixtures which were among the 15 samples tested positive for

Table 4. Results of the dye-based real-time PCR quantitative assay for the binary mixtures

Binary mixture (w/w) ^a	Mean Ct value ^b	SD ^c	Number of positive replicates
0.001 % pork in pork-chicken	28.76	7.9E-2	3/3
0.01 % pork in pork-chicken	28.30	5.6E-2	3/3
0.1 % pork in pork-chicken	27.11	1.6E-1	3/3
1 % pork in pork-chicken	23.34	4.2E-2	3/3
10 % pork in pork-chicken	19.73	1.6E-2	3/3

^aThe binary mixtures were prepared from raw meat bought from local supermarkets.

^bThe Ct values are the mean of replicate assays (n = 3).

^cSD – standard deviation.

^dThe concentration was determined based on standard curve.

porcine DNA. The Ct values of the pork-chicken binary mixtures ranged from 19.73 to 28.76. Notably, the Ct value decreased as the concentration of porcine DNA increased (Table 4). Based on this result, we claimed that detection of as low as 0.001 % (w/w) pork in pork-chicken binary mixture by the proposed assay.

The detection of porcine DNA was achievable under 40 minutes when run with the 7500 Fast Real-Time PCR System (ABI). Nevertheless, 10 ng/μL porcine DNA was readily detected at 11.86th cycle which meant that its presence could be detected much earlier.

The newly developed dye-based qPCR assay in this study showed several merits over other published dye-based qPCR assays. It showed higher sensitivity compared to other probe-based qPCR studies (Ali *et al.*, 2012; Cai *et al.*, 2012; Cammà and Domenico, 2012; Demirhan *et al.*, 2012; Yusop *et al.*, 2012; Iwobi *et al.*, 2015; Kim *et al.*, 2016), in dye-based qPCR study (Soares *et al.*, 2013), and the end-point PCR studies (Amaral *et al.*, 2014; Karabasanavar *et al.*, 2014; Kim *et al.*, 2016). Therefore, this assay is faster and a relatively sensitive alternative. It can also detect porcine adulteration in foods containing varieties of meat species.

Conclusions

Due to the health, cultural and religious importance of unadulterated food, for example the gravity of adulteration and mislabelling in halal foods, a fast and easy method of detection is needed. The real-time PCR can be used to detect and quantify porcine DNA in raw and highly processed food samples. In this study, we reported a novel dye-based qPCR assay for rapid and sensitive detection of porcine DNA in both raw and processed samples. This assay detected as low as 0.00001 ng/μL porcine DNA and 0.001 % (w/w) porcine adulteration in raw meat. It successfully detected 10 ng/μL porcine DNA less than 40 minutes. Therefore, the assay presented itself as a fast and reliable dye-based qPCR assay for porcine DNA detection and quantification.

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Compliance with Ethical Standards

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