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Development and Evaluation of Nested PCR for Specific Identification of Swine-Derived Products in Processed Food and in Animal Feed Concentrates

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Abstract: A nested Polymerase Chain Reaction (PCR) assay for specific identification of pork or swine-derived products in processed food and in animal feed concentrates was developed and evaluated. The mitochondrial cytochrome-b (mtcyt-b) gene was used as a target DNA for PCR amplification. Two pairs of primers (PSL1 and PSR2) and (PSL3 and PSR4), were used for the nested PCR in two amplification steps. First the outer pair of primers (PSL1 and PSR2), derived from a highly conserved region of swine mtcyt-b gene, produced a 1055 base pair (bp) PCR amplicon from swine DNA. Amplification products were visualized on ethidium bromide-stained agarose gels from 100 fg of swine DNA equivalent to 1000 copies of mtcyt-b gene. The nested primers (PSL3 and PSR4) produced a 361 bp PCR product, internal to the annealing sites of primers (PSL1 and RSR2). The nested amplification confirmed the identity of the primary amplified PCR product and increased the sensitivity of the PCR assay. The nested PCR with ethidium bromide-stained agarose gels detected the amount of as little as 0.001 fg of DNA (equivalent to a single copy of Swine-mtcyt-b gene). The specificity studies indicated that neither the primary 1055 bp nor the nested 361 bp PCR products were detected from DNA extracted from a variety of other animal species including, sheep, goat, cattle, deer, camel, horse, donkey, chicken and fish. Application of this nested PCR to processed food including, fresh pork, smoked ham, marinated pork, canned luncheon, pet's food, poultry feed resulted in amplification of the swine specific PCR products. The described nested PCR provides a valuable tool to authenticate the presence of swine-derived product in processed food and in commercial animal feed concentrates.

Key words: Poly Chain Reaction (PCR), swine-derived products, DNA, processed feed conecntrates

INTRODUCTION

Pork consumption is common worldwide but most religious countries are adamants about importing processed food containing pork or swine-derived products. Therefore, development of assays to trace the origin of materials used in processed products would be advantageous in quality control in food industry[1] . The quality control requires that the origin of materials used in processed food be labeled on the products. Therefore, in most countries, regulatory officials require that all ingredients of specified food be labeled on the product for verification. Fresh pork is a protein in nature, which could very easily be detected by immunologic assays. However, it is rather difficult to identify cooked, smoked, marinated or dried pork by immunological assays due to protein denaturing. It is, therefore, becoming a necessity to develop a molecular diagnostic technique to verify the status of swine protein in specified food. It is well documented that nucleic acid hybridization and sequencing techniques have been successfully applied for animal species identification in human food[2]. However, these methods are tedious, laborious and time consuming. Application of the Polymerase Chain Reaction (PCR) and its adaptation for food analysis have proliferated for the examination of food ingredients. These new approaches are alternatives to immunological and DNA hybridization methods. Recently, we described a nested Polymerase Chain Reaction (PCR) assay, for detection of ruminant-derived products in commercial animal feed concentrates, using the mitochondrial cytochrome-b (mtcyt-b) gene as the target DNA[3]. Specific identification of sheep meat or sheep-derived products, in processed human food or in animal feed concentrates, was also described based on the ovine mtcyt-b genome sequence analysis[4]. We have also

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described a reliable, rapid, sensitive and specific PCR assay for specific identification of goat-derived products in processed food and in animal feed concentrate[5]. Simultaneous detection and differentiation of ovine and bovine-derived materials was made possible using a multiplex PCR assay[6].

Because of the stability of the DNA, diagnostic assays, which make use of DNA in the product, can be used to authenticate the animal species utilized in the processed human food or in animal feed concentrates. The objective of the present study was to develop a rapid, sensitive and specific method for detection of pork or swine-derived products in processed food and in commercial animal feed concentrates using nested PCR amplification technology.

MATERIALS AND METHODS

Collection of blood samples: Blood samples were collected in clean sterile vacutainers, containing ethylene diamine tetra acetic acid (EDTA), from different animal species including pigs, sheep, goats, cattle, deer, camel, horse, donkey, chicken and Fish. The blood samples were used for DNA extraction. The extracted DNA was used as the target DNA for PCR amplification.

Extraction of DNA from blood samples: Whole blood was used for extraction of DNA using the commercially available QIAamp blood kit (QIAGEN Inc. Chatsworth, CA) according to the manufacturer's instructions. Briefly, 200 μL of whole blood, 20 μL of proteinase K stock solution and 200 μL of lysing buffer were pipetted into 1.5 mL eppendorf tube and the mixture was incubated at 37°C for 30 min and then at 70°C for 10 min. 200 μL of absolute ethanol was added to the sample and the mixture was mixed by vortexing and spinning. The mixture was transferred to the QIAspin column and placed in a clean 2 mL collection tube and centrifuged at 8000 rpm for 1 min. The QIAspin column was washed twice using 500 μL of washing buffers W1 and W2, respectively, by spinning for 1 minute. The QIAamp spin column was then placed in a clean 1.5 mL eppendorf tube and the DNA was eluted with 200 μL double distilled water preheated at 70°C. Maximum DNA yield was obtained by spinning at 12,000 rpm for 1 min. The DNA concentration was determined by spectrophotometer at 260 nm wave length. Five μL of the extracted DNA was used in the PCR amplification.

Extraction of DNA from processed pork: Swine-derived products used in this study include blood; fresh pork and processed pork including cooked, smoked, marinated, luncheon and microwave-cooked pork. Extraction of DNA from cooked, smoked and marinated pork was performed basically as described for the whole blood extraction procedure[3]. The processed pork was further treated by freezing and thawing and finally incubated at 70°C for 10 min to lyse the content of the food sample. The insoluble component of the food sample was discarded by spinning at 8,000 RPM for 1 min at room temperature. Two hundred μL of the supernatant was used for extraction of swine DNA using the QIAamp blood kits.

Extraction of DNA from animal feed concentrates: Animal feed concentrates including dog food, pet food and commercial cattle feed concentrates were minced and 0.5 gm of the feed concentrate was transferred to 1.5 eppendorf tube for DNA extraction. Details of DNA extraction was described previously by Aradaib *et al.*[6]. Briefly, 200 μL of digestion buffer (50 μL of 10% SDS, 5 μL of 20 mg μL of protinase K and and 130 μL of 0.1 M Tris buffer pH 8.0) were added to the eppendorf tube containing the animal feed. The feed concentrate was mixed by vortexing and incubated at 37°C for 1 hour and then at 70°C for 1 hour. The insoluble component of the food sample was discarded by spinning at 6,000 RPM for 1 min at room temperature. Two hundred μL of the supernatant was used for extraction of swine DNA using the QIAamp blood kits as described above.

Selection of the primers for PCR amplification: For the first amplification step, a pair of primers was selected from the published sequences of the swine mtcyt-b gene and used in the PCR assay[14]. Primers (PSL1 and RSR2) were selected for the synthesis of the primary swine-specific PCR product. Primer PSL1 included bases 63-84 of the positive sense strand (5)-CCC AGC CCC CTC AAA CAT CTC A. PSR2 included bases 1092-1117 of the complementary strand (5)-CGA TGA TGC TAG TGA TTG GTA TCA AT. Using primers PSL1 and PSR2, the primary PCR amplification would result in amplification of a 1055 bp PCR product from swine DNA.

For nested PCR amplification of swine DNA, a pair of internal primers (PSL3 and PSR4) was used in a nested format. The nested primers were designed from the same swine mtcyt-b DNA sequence, internal to the annealing sites of PSL1 and PSR2. PSL3 included bases 228-251 of the positive sense strand (5)-ATG AGT TAT TCG CTA TCT ACA TGC. PSR4 included bases 566-588 of the complementary strand (5)-ATG TAC GGC TGC GAG GGC GGT AA. The nested primers resulted in amplification of a 361 bp PCR product, internal to the annealing sites of primers PSL1 and PSR2. All primers were synthesized on

a DNA synthesizer (Milligan/Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions. The primary and the nested pairs of primers were used at a concentration of 20 picograms per microliter (20 pg μL^{-1}).

Polymerase chain reaction: A stock buffered solution containing 250 μL 10X PCR buffer, 100 μL of $MgCl_2$,12.5 μL of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 mL eppendorf tube. The primers were used at a concentration of 20 pg μL^{-1} and double distilled was added to bring the volume of the stock buffer solution to 1.5 mL. Two μL of the primers, 5.0 μL of the target DNA and 42 μL of the stock solution were added onto 0.5 mL PCR tubes and mixed by vortexing. One μL of Taq DNA polymerase (Perkin Elmer) at a concentration of 5.0 U μL^{-1} were used. All PCR amplification reactions were carried out in a final volume of 50 μL. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by 40 cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 μL from each PCR containing amplified product were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the swine-specific PCR products were easily identified following visualization under UV light.

Nested Polymerase Chain Reaction (nPCR): For the nested PCR amplification, 2.0 μL of the primary amplified product produced by PSL1 and PSR2 were transferred to 0.5 mL PCR tube containing (2 μL of nested primers and; 42 μL of stock PCR buffer and Taq DNA polymerase was used at a concentration of 5.0 U μL^{-1}. The nested pair of primers (PSL3 and PSR4) was expected to amplify a 361 bp PCR product, internal to the annealing sites of primers PSL1 and PSR2. All PCR amplifications were carried out in a final volume of 50 μL. The thermal cycling profiles were as follows: A 2 min incubation at 95°C, followed by 30 cycles of 94°C for 1 min, 57°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 μL from each PCR containing amplified products were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the swine-specific PCR products were easily identified following visualization under UV light.

RESULTS

The described PCR-based assay reproducibly allowed sensitive and specific identification of swine-derived product used in this study. Using the first pair of primers (PSL1 and PSR2), the swine-specific 1055 bp PCR product was visualized on ethidium bromide-stained gels from 100 fg of swine DNA extracted from blood samples of pigs (Fig.1). However, the nested primers (PSL3 and PSR4) produced a 361 bp swine-specific PCR product from 0.001 fg of swine mtcyt-b DNA target, equivalent to a single copy of mtcyt-b gene (Fig. 2). The specificity studies indicated that the amount of 1.0 pg DNA from a variety of different animal species including sheep, goat, cattle, camel, deer, horse, donkey, chicken and fish failed to produce the specific PCR product (Fig. 3). The described PCR assay resulted in detection of the 361 bp nested PCR products from DNA extracted from a variety of swine-derived products including blood, fresh pork, cooked pork, marinated ham, luncheon, smoked ham, pig tallow and fats (Fig. 4). Application of this nested PCR assay resulted in detection of the specific 361 bp PCR product from different preparation of animal feed including, dog food, canned pet's food, poultry feed (Fig. 5).

DISCUSSION

Breeding of pigs and consumption of pork is not uncommon in some Islamic countries. Therefore, the development of a molecular diagnostic technique for identification of biomaterials from complex sources would be advantageous in a variety of circumstances including comparative genomics and investigative forensics. In a previous report, differentiation of sheep and goat meat was shown by comparison of their nucleotide sequences through direct sequencing techniques and digestion of PCR product with restriction endonuclease enzymes. Analysis of the PCR fragment with endonuclease enzymes to detect Restriction Fragment Length Polymorphisms (RFLPs) was also described. This analysis is useful to differentiate species instead of sequencing the common fragment[7,8,9]. Application of PCR-RFLP technique was described for differentiation of meat species particularly food animals[10,11,12]. In a previous study, pork was differentiated from sheep and goat meat by comparison of their nucleotide sequences through direct sequencing techniques and digestion of PCR product with restriction endonuclease enzyme[7,13,14]. However, this method is tedious, laborious, time consuming and expensive. The nested PCR amplification technology, described in this study, provides a simple, rapid and sensitive method for

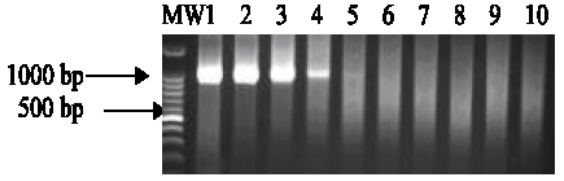

Fig. 1: Sensitivity of the PCR assay for the detection of the swine-specific 1055 bp PCR product, using primers PSL1 and PSR2). Visualization of the 1055 bp PCR product on ethidium bromide-stained agarose gel from 100fg of swine DNA. Lane M: molecular weight marker (100 bp ladder); lanes1-10: swine DNA at concentrations of 1ng, 100, pg, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg, o.o1 fg and 0.001 fg, respectively

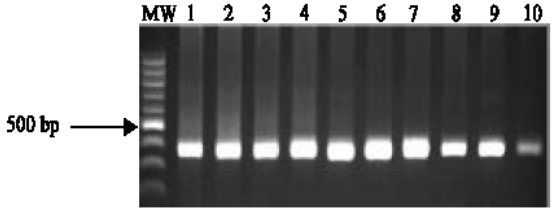

Fig. 2: Nested amplification of the 361 bp PCR products from the primary PCR product. The nested PCR amplification detected as little as 0.001 fg of swine DNA. Lane M: molecular weight marker; lanes1-10: swine DNA at concentrations of 1ng, 100,pg, 10pg, 1pg, 100fg, 10 fg, 1fg, 0.1fg, o.o1 fg and 0.001 fg , respectively

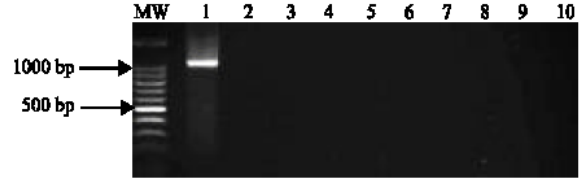

Fig. 3: Specificity of the nested polymerase chain reaction for specific identification of swine DNA using primers (PSL3 and PSR3). Amplification product was not detected from other DNA of other animal species including, sheep, goat, cattle, camel, horse, donkey, chicken and fish. Lane MW: molecular weight marker; Lane 1: 1 pg swine DNA (positive control); Lane 2-10: DNA extracted from sheep, goat, cattle, camel, deer, horse, donkey, chicken and fish; respectively

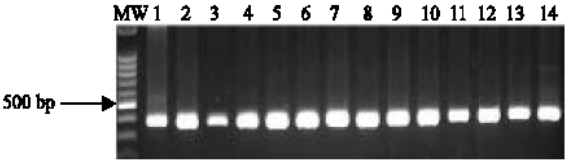

Fig. 4: Application of PCR for direct detection of the specific 361 bp PCR product from swine-derived product in processed food and in anima feed concentrates. Lane 1: 1 pg swine DNA (positive control); lane 2: marinated ham; Lane 3: microwaved ham for 15 min; lane 4: smoked ham; lane 5: boiled ham supernatant; Lane 6: boiled ham sediment; lane 7-8: frozen ham; lane 9: cooked luncheon; lane 10: boiled pork; Lane 11: boiled luncheon sediment; lane 12: frozen pork; Lane 13: canned pork; lane 14: pig fat (tallow) of primers (PSL2and PSR4)

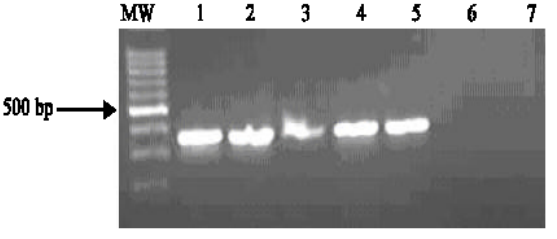

Fig. 5: Application of PCR for direct detection of the specific 361 bp PCR product from swine-derived product in commercial animal feed concentrates. Lane 1: swine blood (positive control); lane 2-3: pets food; Lane 4-5; dog food; Lane 6-7: bovine DNA (negative controls)

detection and identification of pork or swine-derived product in processed food and in animal feed concentrates. DNA extraction was a simple procedure that takes only half an hour using QIAamp extraction kit. The time required for the primary and the nested amplifications was approximately 4 h. The electrophoresis, staining of the agarose gel with ethidium bromide and visualization of the specific PCR products usually takes one hour. Confirmatory results of submitted samples could be obtained within the same working day. The nested amplification increased the sensitivity of the PCR assay particularly, when the concentration of the swine DNA in the sample is less than 100 fg, which is beyond the detection limit of the agarose gel electrophoresis. The described PCR assay detected as little as 0.001 fg of swine DNA which is equivalent to a single copy of swine

mtcyt-b gene. To our knowledge, this nested PCR assay provides the most sensitive method for detecting pork or swine-derived contaminants in a variety of complex biomaterials. The specificity studies indicated that the primary 1055 bp PCR product was not amplified even from a relatively high concentration of 1.0 ng of DNA extracted from ruminant species (sheep, goat, cattle and deer) or from other animal species including, camel, horse, donkey, chicken and fish, under the same stringency condition described in this study. Using the pair of internal primers, the nested amplification confirmed the identity of the primary amplified product and thereby avoiding the hybridization confirmation procedure, which is cumbersome and usually takes overnight.

It is worth mentioning that the nested PCR, described in this study, could be used as a valuable tool in forensic veterinary medicine to authenticate the presence of pork or swine-derived product in processed food or in animal feed concentrates to resolve the problem of religious restrictions.

In a previous report, it has been shown that heat treatment or cooking of meat of various animal species including pork resulted in decreased sensitivity of the PCR assay[15-17]. Nevertheless, in the present study, treatment of pork with high temperature, such as cooking in the microwave for 15 minutes, did not affect the detection limit of the nested PCR assay[17,18]. This could be attributed to the different methods of DNA extraction, which subsequently resulted in different DNA yield from heat treated samples. Thus, application of this PCR could be useful for detection of heat-treated products including microwaved cooked pork and smoked luncheon. Detection of animal derived products by PCR has been proliferated because of it's simplicity, rapidity, reliability, sensitivity and specificity[19-24]. In previous studies, different genes were targeted for PCR amplifications including growth hormone gene; mtcyt-b gene and interspersed repetitive elements[21,22]. In the present study, selection of the primers was based on the observation that the mtcyt-b gene has high copy numbers (hundred to thousand copy numbers per cell). In addition, the occurrence of conserved regions within the mtcy-b gene provides high sensitivity for the nested PCR[12,13,14]. More over, the described nested PCR assay does not require sophisticated laboratory equipments such like DNA sequencer and hybridization facilities. Further more, analysis of the PCR fragments with endonuclease enzymes to detect restriction fragment length polymorphisms (RFLPs) was not employed in this study. This analysis is also useful to differentiate animal species. However, PCR-RFLPs is time consuming and expensive. The nested PCR, described in this study, could be used as a valuable tool in forensic veterinary medicine to authenticate the presence of pork in complex biomaterials in processed food or in animal feed concentrates to resolve the problem of religious restrictions.

It is worth mentioning that complex biomaterials containing swine tissues are accepted as a source of protein in animal feed concentrates. Nevertheless, in some countries, religious people particularly, Muslims and Jewish are very adamant about consumption of pork or even feeding swine-derived materials to animals. This concern would require strict screening for swine product in human food and animal feed concentrates, which could be provided by the above described PCR-based assay.

This nested PCR assay should provide verification of the swine protein status of specified processed food or animal feeds concentrate.

CONCLUSIONS

The described nested PCR assays should be used as simple and rapid methods for detection and specific identification of swine-derived products in processed food and animal feed. In addition, application of this assay would be advantageous in comparative genomics and investigative forensics and can be recommended in quality control departments.

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