ANIMAL SPECIES IDENTIFICATION USING MITOCHONDRIAL CYTOCHROME B GENE

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ABSTRACT

The polymerase chain reaction PCR (Conventional and real time) were applied to identify seven animals species (goat, cattle, sheep, pig, donkey, cat and dog).Species-specific primers were used from mitochondrial cytochrome b gene. Identification was possible for fresh, heat treated (at 120°C for 30 min) and putrefied (for 7 days) meat. Multiplex PCR helped for rapid detection and identification of meat species. PCR products showed species-specific DNA fragments of 157, 274, 331, 398, 493, 672 and 808 bp and Ct value (15.79, 17.55, 17.09, 15.94, 17.28, 19.54 and 15.79) when used real time PCR from goat, cattle, sheep, pig, donkey, cat and dog meats respectively.

INTRODUCTION

The stability of DNA and PCR techniques have many potential advanges over protein based techniques such as ELISA which depend primarily on protein detection of the antibodies **(Asensio et al., 2008)**. PCR analysis of species-specific mtDNA sequences is the most common method currently used for species identification **(Cann et al., 1987 and Parodi et al. 2002)**. Furthermore, the application of DNA method based on mtDNA facilitates the PCR amplification in cases where the availability of DNA template after its extraction from cells is insufficient for detection , as mtDNA is several fold more abundant that of the nuclear genome; each mitochondria is estimated to contain 2-10 mtDNA copies and each cell carries multiple numbers of mitochondria, depending on the tissue and species **(Wiesner et al., 1992)**. Mitochondrial DNA evolves much faster than nuclear DNA and presents more sequence diversity, thus facilitating the identification of closely related species **(Wolf et al., 1999)**. The characteristic high copy number, material inheritance and high degree of sequence variability make mtDNA a powerful tool for forensic identification **(Rastogi et al., 2007)**. PCR based techniques have proved to be reliable, sensitive and fast **(Fajardo et al., 2007, Kesman et al., 2007 and Martin et al., 2007)**.

Conventional PCR detected meat species prepared at high temperature from DNAs of heat treated meat at 120ºC temperature. Heat treated did not affect the DNA extraction or the profiles generated **(Guoli et al., 1999 and Hopwood et al., 1999)** DNA has the advantage of being a relatively stable molecule under heat **(Chen et al., 2004 and Lanzilao et al., 2005)**. Protein are often less sensitive or

even may fail in the analysis of heat treated materials **(Real et al., 2008)**.

The analytical methods employed in species identification in mixture should be rapid and easy to perform, without being cost prohibitive so multiplex PCR improved that **(Mackie et al., 1999; Matsunga et al., 1999; Bottero et al., 2003 and Dalmasso et al., 2004)**.

Real-time PCR used for the identification of animal amplifying mtDNA genes as cytochrome b gene **(Dooley et al., 2004; Hird et al., 2004 and Chisholm et al., 2005)**. The main advantages of real-time PCR technology are the capacity to discriminate the DNA of origin without the need for any additional time consuming and laborious steps (post PCR process), and the possibility to perform quantitative measurements through the use of a fluorometer built into the thermal cycler that generates a thermal denaturation curve of the amplified product. This is very useful in confirming the identity of an amplicon. Unlike gel electrophoresis, melting curve analysis can distinguish products of the same length but different GC/AT ratio **(Ririe et al., 1997 and Welleret al., 2000)**. This research aimed to the identification of animal species from fresh, heat treated, putrefied and even in mixture.

MATERIAL AND METHODS

Samples:

Thirty five meat samples (musculoskeletal tissue). Five samples from each species were collected. goat (Capra hircus), cattle (Bos tauros), sheep (Ovis aries) were collected from slaughterhouse. Pig (Sus scrofa) samples were collected from meat markets. Donkey (Equus asinus) samples were collected from Mansoura Zoo. cat (Felis catus) and dog (Canis familiaris) samples were collected from Faculty of Veterinary Medicine, Mansoura University. The samples were kept at -20°C till extraction.

DNA extraction:

Total genomic DNA isolated by using Axy-Prep Multisource Genomic DNA Miniprep Kit (cat. no. AP-MN-GDNA-50, Axygen Bioscience, CA, USA) according to the manufacture's instructions.

Species-specific primers :

Based on the mitochondrial cytochrome b gene as it was summarized in Table 1.

Table 1: Species-specific primers

Primer	Primer sequence (5` to 3`)	Reference
SIM F	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA	Matsunga et al., 1999
Goat R	CTC GAC AAA TGT GAG TTA CAG AGG GA	
Cattle R	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG	
Sheep R	CTA TGA ATG CTG TGG CTA TTG TCG CA	
Pig R	GCT GAT AGT AGA TTT GTG ATG ACC GTA	
Donkey R	CTC AGA TTC ACT CGA CGA GGG TAG TA	
Cat F	CTC ATT CAT CGA TCT ACC CA	Abdulmawjood et al., 2003
Cat R	GTG AGT GTT AAA ACT AGT ACT AGA AGA	
Dog F	GGA GTA TGC TTG ATT CTA CAG	
Dog R	AGA AGT GGA ATG AAT GCC	

Polymerase chain reaction (PCR):

PCR amplification was carried out in 25 µl of 2 µl primer(F and R) (10 pmol/ µl) and 1 µl DNA template (5 ng /µl).

The PCR was carried out in thermal cycler (Gen Amp 2400 Appled Biosystem, USA for traditional PCR and stratagene Mx3000p QPCR for real-time PCR) with the following program : initial denaturation at 95°C for 5 min 30 cycles consisted of (denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 min) with final extension at 72°C for 7 min. Following amplification 10 µl PCR products were electrophoresed on 1 % agarose gel containing ethidium bromide solution (2µl/100 ml) at constant voltage 80 V for 30 minutes in 1X TBE buffer.

RESULTS

PCR profiles of fresh meat samples :

DNA extracted from 20 mg of fresh meat samples of seven species and were used as a template for PCR. The PCR products are shown in Fig (1).

PCR profiles of heat treated meat samples :

Meat samples of seven species were subjected to heat treatment to simulate cooking at 120°C for 30 minutes. DNA extracted from 20 mg of muscle samples was used as a template for PCR. The PCR products are shown in Fig (2).

PCR profiles of putrefied meat samples:

PCR profile of cyt b gene generated from DNA extracted from putrefied meat the same as DNA of fresh meat samples. PCR using mt yt b gene species-specific primers successfully gave amplification from meat samples that were putrefied for 7 days indicating that purification did not inhibit efficiency of amplification of cyt b gene of different species in PCR products are shown in Fig (3).

Multiplex PCR :

A multiplex PCR was designed by mixing all primers in a single reaction and due to having confirmed species specificity of each primer independently. Initially, all the primers were mixed in equal proportion (1: 1: 1: 1: 1: 1: 1: 1: 1: 1: 1) for SIM: goat: cattle: sheep: pig: donkey: cat F: cat R: dog F: dog R and DNA mixed in equal proportion (1: 1: 1: 1: 1: 1: 1) for goat: cattle: sheep: pig: donkey: cat: dog then diluted to 50 times. This multiplex PCR was tested on DNA sample from each species one by one and DNA mixtures of seven animals species.

The multiplex PCR amplified fragments specific to each species producing characteristic band pattern on agarose gel electrophoresis when run on single species and a multiple band pattern when run on DNA mixture due to the primers retained the same specificity and absence of cross reaction. PCR amplicons that shown in Fig (4).

Real Time PCR :

The mitochondrial cytochrome b gene was used with SYBR Green. Comparative between the seven animal DNA indicated that the present of specific amplification curve for each one with different Ct value (15.79, 17.55, 17.09, 15.94, 17.28, 19.54 and 15.79) from goat, cattle, sheep, pig, donkey, cat and dog meats respectively which specific for each animal DNA.

DISCUSSION

The successful amplification of cyt b gene fragment in all fresh meat species to confirm the product size in various species. All the independent (species wise) PCRs amplified the fragments of expected size, i.e. 157 bp for goat,274 bp for cattle, 331 bp for sheep, 398 bp for pig and 439 bp for donkey **(Matsunaga et al., 1999, Obrovska et al., 2002, Jain et al., 2007)** 762 bp for cat and 808 bp for dog **(Abdulmawjood et al., 2003, Abdel-Rahman et al., 2009)**.

Species identification of cooked meat is warranted. The processing technology (salting, drying, smoking, and cooking) applied during the manufacture of meat products may affect to different extents to the integrity of the extractable DNA. Heat treatments are those steps, which mainly affect the quality of DNA causing its degradation into small size fragments **(Dias Neto et al., 1994 and Martinez and Y. Man, 1998)** For this reason, meat samples were heat treated in the present study at 120°C in autoclave for 30 minutes to simulate cooking. Proper cooking was evident from discolored meat. These findings confirmed the results obtained by **(Matsunaga et al., 1999)** who reported the similar result.

In forensic investigation carcasses putrefied or meat samples are brought for speciation after one or two days of slaughter under unpreserved conditions. It was attempted to study the affect of putrefaction on PCR pattern. PCR successfully amplification of mitochondrial cyt b gene from meat samples that were putrefied even for seven days **(Mudiyanselage, 2009)**.

Multiplex PCR is a good tool to identify and distinguish between animal's meat species through the mitochondrial cytochrome b gene and become useful tool for animal species identification (goat, cattle, sheep, pig, donkey, cat and dog) in forensic PCR. As the new development of molecular technology, identification by the species-specific diagnostic PCR only needs a single specific-reaction step. Additional, combining the multiplex PCR technology, by running a single PCR step reaction the fragments of positive control and the species-specific fragments can be produced at a time. This avoids the possible false negative results caused by some mistake from the experimenters. So the method of species identification by multiplex PCR has been applied successfully in many species**(Dizon et al., 2000, Hare et al., 2000, Pank et al.,2001, Bottero et al., 2002, 2003, Shivji et al., 2002, Chapman et al., 2003).** The primers mixture produced the respective pattern for each species with the DNA mixture or with DNA of each animal species due to the primers specificity and absence of cross reaction.

Meat samples were declared to contain specific DNA when analyzed in the species-specific PCR system produced specific amplification products from specific primer and DNA sequence for that produced specific Ct value. This is the main reason why Ct is a more reliable measure of starting DNA copy number than an end point measurement of the amount of accumulated PCR product **(Lahiffet al., 2002)**.

It could be concluded that:

Mitochondrial cytochrome b gene improved to be a good tool for forensic animal identification and speciation.

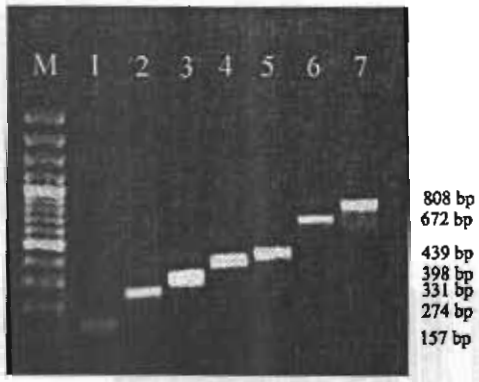

Fig (1) : Electrophoretic analysis of PCR products from fresh meat samples of 7 animals species. (M) marker 100 bp ladder. (1) goat, (2) cattle, (3) sheep, (4) pig, (5) donkey, (6) cat, (7) dog.

Fig (3) : Electrophoretic analysis of PCR products from putrefied meat samples of 7 animals species. (M) marker 100 bp ladder. (1) goat, (2) cattle, (3) sheep, (4) pig, (5) donkey, (6) cat, (7) dog.

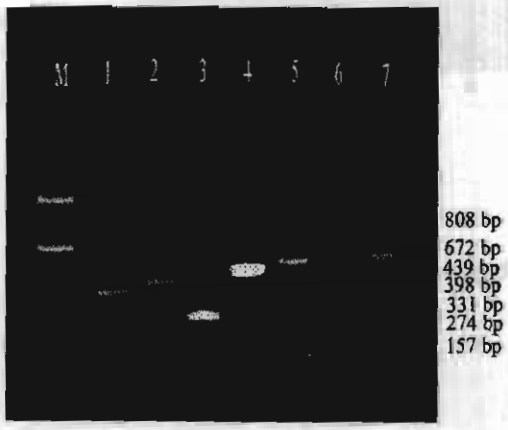

Fig (2) : Electrophoretic analysis of PCR products from heat treated meat samples of 7 animals species. (M) marker 100 bp ladder. (1) cattle, (2) sheep, (3) goat, (4) pig, (5)donkey, (6)dog, (7) cat.

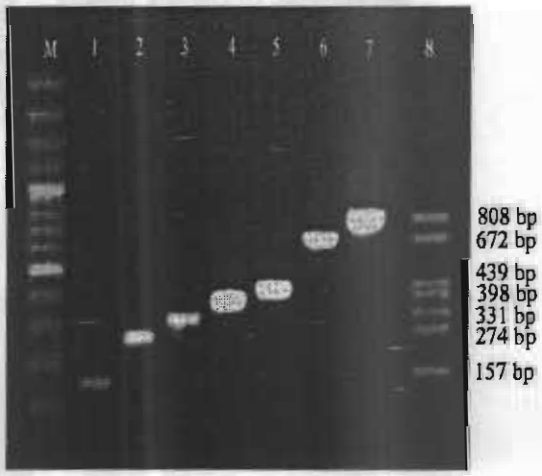

Fig (4) : Electrophoretic analysis of multiplex PCR products from meat samples of 7 animals species. (M) marker 100 bp ladder. (1) goat, (2) cattle, (3) sheep, (4) pig, (5) donkey, (6) cat, (7) dog, (8) DNA mixture of seven animal species.

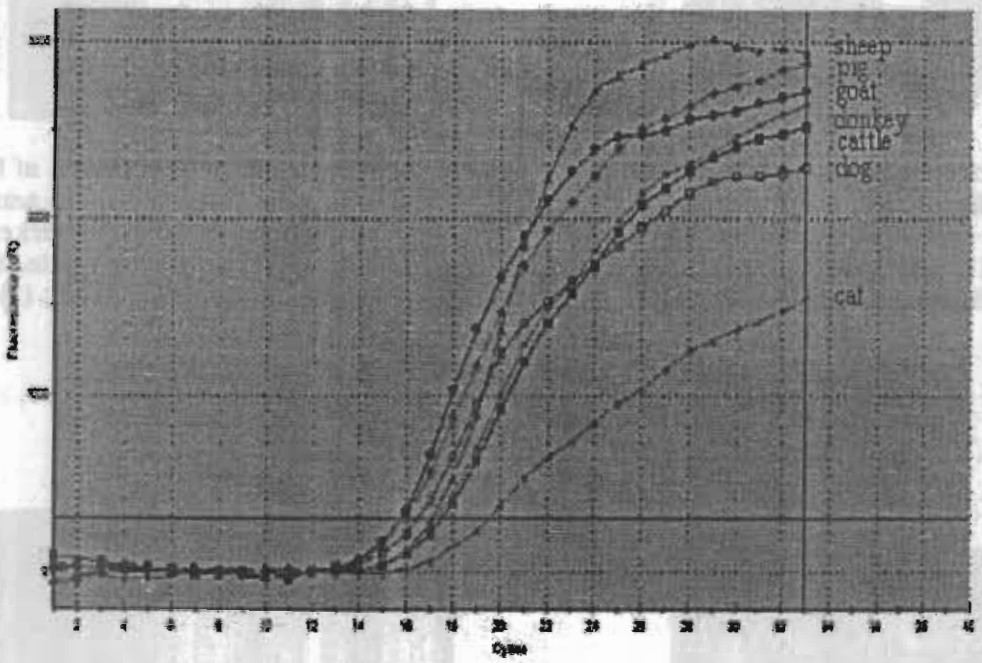

Fig (5) : Real Time PCR amplification curve of cyt b gene fragment of seven animals meat DNA.

REFERENCES

Abdel-Rahman, S. M.; El-Saadani, M. A.; Ashry, K. M.; Amany, S. and Haggag, A. S. (2009) : Detection of Adulteration and Identification of Cat's, Dog's, Donkey's and Horse's Meat Using Species-Specific PCR and PCR-RFLP Techniques. Australian J.of Basic and Applied Sci. 3(3), 1716-1719.

Abdulmawjood, A.; Schonenbrucher, H. and Bulte, M. (2003) : Development of a Polymerase Chain Reaction System for the Detection of Dog and Cat Meat in Meat Mixtures and Animal Feed. J. of Food Sci. 68, 1757-1761.

Asensio, L.; Gonzalez, I.; Pavon, M. A., Gracia, T. and Martin, R. (2008) : An indirect ELISA and a PCR technique for the detection of grouper (Epinephelus marginatus) mislabeling. Food Additives and Contaminants: Part A Chemistry, Analysis, Control, Exposure and Risk Assessment. 25 (6), 677-683.

Bottero, M. T.; Civera, T.; Anastasio, A.; Turi, R. M. and Rosati, S. (2002) : Identification of cow's milk in "buffalo" cheese by duplex polymerase chain reaction. J. Food Prot. 65, 362-366.

Bottero, M. T.; Civera, T.; Nucera, D.; Rosati, S.; Sacchi, P. and Turi, R. M. (2003) : A multiplex polymerase chain reaction for the identification of cows' goats' and sheep's milk in dairy products. Int. Dairy J. 13, 277-282.

Cann, R. L.; Stoncking, M. and Wilson, A. C. (1987) : Mitochondrial DNA and human evolution. Nature. 325, 31-36.

Chapman D. D., Abercrombie D. L., Douady C. J., Pikitch E. K., Stanhope M. J. and Shivji, M. S. (2003). A streamlined, bi-organelle, multiplex PCR approach to species identification: Application to global conservation and trade monitoring of the great white shark, carcharodon carharias. Conserv. Genet. 4, 415-425.

Chen, F. C., Hsieh, Y. H. P. and Bridgman, R. C. (2004) : Monoclonal antibody-basd sandwish enzyme-linked immunoabsorbent assay for sensitive detection of prohibited ruminant proteins in feedstuffs. J. Food Prot. 67, 544-549.

Chisholm, J.; Conyers, C.; Booth, C., Lawley, W. and Hird, H. (2005) : The detection of horse and donkey using real-time PCR. Meat Scie. 70, 727-732.

Dalmasso, A.; Fontanella, E.; Piatti, P.; Civera, T.; Rosati, S. and Bottero, M. T. (2004) : A multiplex PCR assay for the identification of animal species in feedstuffs . Molecular and Cellular Probes. 18, 81 -87.

Dias Neto, E.; Caballero, O. L.; Vidigal, T.; Pena, S. and Simpson, A. (1994) : Producing randomly amplified polymorphic DNAs from degraded DNA. In workshop of Genomic Fingerprinting. Madrid, Spain: Instituto Juan March de Estudios Investigaciones. 20, 94-95.

Dizon, A.; Baker, C. S.; Cipriano, F.; Lento, G.; Palsboll, P. and Reeves, R. (2000). Molecular genetic identification of whales, dolphins and porpoises. In: Proceedings of a workshop on the forensic use of molecular techniques to identify wildlife products in the market-place. La Jolla, California: NOAA Technical Memorandum NMFS NOAA-TM-NMFS-SWFSC-286.

Dooley, J. J.; Paine, K. E.; Garrett, S. D. and Brown, H. M. (2004) : Detection of meat species using TaqMan real-time PCR assays. Meat Scie. 68,431-438.

Fajardo, V.; González, I.; Lopéz, I. C.; Martín, I.; Rojas, M.; García, T.;

Hernández, P. E. and Martín, R. (2007) : PCR identification of meats from chamois (Rupicapra rupicapra), pyrenean ibex (Capra pyrenaica), and mouflon (Ovis ammon) targeting specific sequences from the mitochondrial D-loop region. Meat Sci. 76, 664-652.

Guoli, Z.; Mingguang, Z.; Zhijiang, Z.; Hongsheng, O. and Qiang, L. (1999) : Establishment of a polymerase chain reaction for the identification of beef. Meat Sci. 51, 233-236.

Hare, M. P.; Palumbi, S. R. and Butman, C. A. (2000) : Single-step species identification of bivalve larvae using mulitiplex polymerase chain reaction. Mar. Biol. 137, 953-961.

Hird, H.; Goodier, R.; Schneede, K.; Boltz, C.; Chisholm, J.; et al. (2004) : Truncation of oligonucleotide primers confers specificity on real time-PCRassays for food authentication. Food Additives and Contaminants. 21(11), 1035-1040.

Hopwood A. J., Fairbrother K. S., Lockley A. K and Bardsly R. G . (1999). An actin gene - related polymerase chain reaction (PCR) test for identification of chicken in meat mixtures. Meat Sci. 53, 227-231.

Jain, S.; Brahmbhatt, M. N.; Rank, D. N.; Joshi, C. G. and Solank, J. V. (2007) : Use of cytochrome b gene variability in detecting meat species by multiplex PCR assay. Indian. of Animal Sci. 77 (9), 880-881.

Kesmen, Z.; Sahin, F. and Yetim, H. (2007) : PCR assay for the identification of animal species in cooked sausages. Meat Sci. 77, 649-653

Lahiff, S.; Glennon, M.; Lyng, J.; Smith, T.; Shilton, N. and Maher, M. (2002) : Real- Time polymerase chain reaction for detection of bovine DNA in meat and bone meal Samples. J. of Food Prot. 65(7), 1158-1165.

Lanzilao, I.; Burgalassi, F.; Fancelli, S.; Settimelli, M. and Fani, R. (2005) : Polymerase chain reaction- restriction fragment length polymorphism of mitochondrial cyt b gene from species of dairy interest. J. of AOAC International. 88, 128-135.

Mackie, I. M.; Pryde, S. E.; Gonzales-Sotelo, C.; Medina, I.; Pe´rez Marti´n, R.; Quinteiro, J.; et al. (1999). Challenges in the identification of species of canned fish. Trends in Food Science & Technology.s 10, 9-14.

Martinez, I. and Yman, I. M. (1998): Species identification in meat products by RAPD analysis. Food Res. Int. 31 (6-7), 459-466.

Martín, I.; García, T.; Fajardo, V.; Lopéz, I. C.; Rojas, M.; Hernández, P. E.; et al. (2007) : Mitochondrial markers for the detection of four duck species and the specific identification o Muscovy duck in meat mixtures using the polymerase chain reaction. Meat Sci. 76,721-729.

Matsunaga, T.; Chikuni, K.; Tanabe, R.; Muroya, S.; Shibata, K.; Yamada, J. and Y. Shinmura. (1999) : A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci. 51(2), 143-148.

Mudiyanselage, D. D. D. N. G. (2009) : An assessment of the impact of environmental factors on the quality of post-mortem DNA profiling. PhD in the Discipline of Anatomical Sci. Faculty of Heleth Sci. The University of Adelaide, Australia.

Obrovska, I.; Steinhauserova, I. and Nebola, M. (2002) : The application of the PCR

method to the identification of meat species. Folia Veterinaria. 46(3), 113-118.

Pank, M.; Stanhope, M.; Natanson, L.; Kohler, N. and Shivji, M. (2001) : Rapid and simultaneous identification of body parts from the morphologically similar sharks Carcharhinus obscurus and Carcharhinus plumbeus (Carcharhinidae) using multiplex PCR. Mar. Biotechnol. 3, 231-240.

Parodi, B.; Aresu, O.; Bini, D.; Lorenzini, R.; Schena, F.; Visconti, P.; Cesaro, M.; Ferrera, D.; Andreotti, V. and Ruzzon, T. (2002) : Species identification and confirmation of human and animal cell lines: a PCR based approach. Biotechniques. 32, 433-440.

Rastogi, G.; Dharne, M. S.; Walujkar, S.; Kumar, A.; Patole, M. S. and Shouche, Y. S. (2007) : Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. Meat Sci. 76, 666-674.

Real, S.; Campanella, A.; Merigioli, A. and Pilla, F. (2008) : Anovel method for species identification in milk and milk-based products. J, Dairy research. 75, 107-112.

Ririe, K. M.; Rasmussen, R. P. and Wittwer, C. T. (1997) : Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry, 245, 154-160.

Shivji, M.; Clarke, S.; Pank, M.; Natanson, L.; Kohler, N. and Stanhope, M. (2002) : Genetic identification of Pelagic shark body parts for conservation and trade monitoring. Conserv. Biol. 16 (4), 1036-1047.

Welleret, S. A.; Elphinstone, J. G.; Smith, N. C.; Boonham, N. and Stead, D. E. (2000) : Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology, 66, 2853-2858.

Wiesner, R. J.; Ruegg, J. C. and Morano, I. (1992) : Counting target molecules by expontial polymerase chain reaction, copy number of mitochondrial DNA in rat tissues. Biochimica et Biophysica Acta. 183,553-559.

Wolf, C.; Rentsch, J. and Hubner, P. (1999) : PCR-FRLP analysis of mitochondrial DNA: A reliable method for species identification . J. Agric. Food Chem.47, 1350-1355.

الملخص العربى

التعرف على أنواع الحيوانات باستخدام ميتوكوندريا سيتوكروم بى چين

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نظرا لانتشار حالات الغش التجاري في اللحوم المختلفة وأيضا لانتشار الأمراض المختلفة التي قد تنقل من حيوان لإنسان عن طريق الخطأ في التصنيع أثناء ممارسة الغش التجاري و أيضا التعرف علي أنواع الجثث المختلفة يعتريها التعفن أو أثناء الكوارث التي يصعب علينا التعرف عليها بمجرد النظر فقد رأينا أن تطبيق الإستعراف علي أجزاء الحيوانات المختلفة بتطبيق تفاعل البلمرة المتسلسل بنوعيه ((Conventional and Real-Time PCR التقليدي و الكمي للتعرف علي سبعة أنواع من الحيوانات و هي الماعز و الأبقار و الأغنام و الخنازير و الحمير و القطط و الكلاب من خلال (Mitochondrial cytochrome b gene) والحامض النووي (DNA) من عينات اللحوم الطازجة و المعاملة حراريا لمدة نصف ساعة عند درجة 120°C و الفاسدة لمدة 7 أيام. حصل علي الحزم المتوقعة من (Conventional PCR) وهي 808 bp , 672 , 439 , 398 , 331 , 274 , 157 و كذلك (C_t) من خلال (Real-Time PCR) وقيمتها 15.79 , 17.55 , 17.09 , 15.94 , 17.28 , 19.45 , 15.79 للماعز و الأبقار و الأغنام و الخنازير و الحمير و القطط و الكلاب كلا علي حده وهذا يساعدنا على سرعة الفصل فى الأنواع المختلفة للحيوانات من فحص أجزائها.