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Identification of meat products by using mitochondrial DNA Hypervariable region

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Abstract
Many techniques were developed to detect and identify the type of meat used in industrial meat products. This issue is very important for many points of view including, health, religion and detection of adulteration. In the present study, we used a simple system for species identification. It is based on size variations of the amplified mitochondrial DNA hyper variable regions among species. DNA samples from meat products and fresh meat control for each species were extracted. Samples collected from market include 10 cow, 10 sheep and 10 chicken products. Amplification of the hyper variable region was done using two primer sets specific for mammals and birds. The differences in the sizes of the polymerase chain reaction (PCR) products compared to a ladder for size identification permitted us to identify species. In case of second band appearance the identification is done by size and confirmed by sequencing. The extra band was cut from gel, purified and sequenced. The obtained sequences were submitted to GenBank sequences database (BLAST analysis) for species identification. The results showed the appearance of single band in case of samples collected from cows and chicken. In case of sheep samples, in addition of the normal sheep band in most samples, some showed an extra band. This band was identified as cow meat which reflects meat mixing.

Keywords: Species identification, universal primers, mitochondrial DNA, hypervariable region

Introduction

There is no doubt that sheep, cow and chicken meat products are becoming more consumable products all over the world, especially in Kingdom of Saudi Arabia. The national companies were not able to provide and fulfill the increase in demand. In order to cover this shortage, it is important to import frozen sheep, cow and chicken and its derivatives [1]. For consumers, it is difficult to detect adulteration in these products like expired date of meat and adulterated constitutes.

Recent food scares (e.g. bovine spongiform encephalopathy avian flu, foot-and-mouth disease), malpractices of some food producers, religious reasons, food allergies and genetically modified organisms (GMOS) have tremendously reinforced public awareness regarding the composition of food products [1]. As labels, do not provide enough guarantees about the true contents of a product, it is necessary to identify and authenticate the components of processed food, thus protecting consumers from illegal substitutions [2].

Food authentication and protection of biodiversity both require reliable and accurate methods for determining without ambiguity, the animal species in a wide array of degraded and processed substrates. Additionally, the development of these methods should protect consumers from frauds, and protect animal species from over-exploitation of illegal trafficking [3,4].

With the recent progress in molecular biology, new methods have been developed on the basis of the genetic differences among species. The cytochrome *b* (CYB), cytochrome oxidase I [5], 12 S ribosomal RNA (rRNA) [6, 7], 16 S ribosomal RNA (rRNA) [8] and hypervariable regions (HVR) are genes in mitochondrial DNA (mt-DNA) have been widely used targets for animal species identification [9].

Mitochondria are sub-cellular organelles containing an extra chromosomal genome that is separated and distinct from the nuclear genome [1]. Mitochondria have a critical role in oxidative phosphorylation process to produce ATP. Mitochondrial DNA (mt-DNA) contains 13 genes that encode 13 polypeptides, 22 t-RNAs, and 2 r-RNAs. It is maternally inherited and does not undergo recombination. It is valuable DNA used for investigating phylogenetic relationship among populations, subspecies, and species [10].

The urgent need for accurate and reliable methods for animal species identification has steadily increased during past decades, particularly with the recent food scares and the overall crisis of biodiversity primarily resulting from the huge illegal traffic of endangered species [1]. A relatively new biotechnological field, known as species molecular identification which is based on the amplification and analysis of DNA offers promising solutions [3].

The aim of this study was focused to identify meat, meat products and authentication in these products in order to protect consumers from illegal substitutions which occurs as a result of survival in the competitive market and make a profit excessive [4].

Materials and Methods

The method used in this study is based on amplification of specific fragment of mitochondrial DNA (hypervariable region) of D-loop by PCR technique [11, 12, 13]. DNA sequencing and the obtained Blast search results are confirmed by using a set of suitable PCR primers [7, 14].

Meat Samples

Biological samples of frozen and canned meat were randomly

collected from different markets in Makkah and Jeddah cites in Saudi Arabia. Ten samples were used from each species i.e., sheep, cows and chicken. For each species, fresh meat was used as a positive control**.** DNA was extracted from meat samples by using QIAamp DNA mini® Kit (250) (Qiagen, cat. Nos.51304 and 51306, www.qiagen.com) following the manufacturers protocol.

Conventional system for species identification

A method was developed using primers and agarose gel electrophoresis. The sequences and priming position of the PCR primers used in this study are shown in Table 1.

The PCR reaction for mammals and birds were carried out as follows (each species in separate reaction): the PCR reaction mixture (25 µl) contained 1 µl of extracted DNA(0.1-1 ng of DNA), 1 µl of each primer (forward and reverse), 9.5 µl of sterilize distal water, 12.5 µl of Master mix (DreamTaq Green PCR Master Mix (2X)- Catalog number: K1081) at 98°C for 30 sec, followed by 35 cycles of 98 °C for 10 sec, 60 °C in mammals and 56 °C in birds for 30 sec, 72 °C for 1 min, and a final extension at 72 °C for 7 min (Table 2). The extraction reagent blank and a positive and negative PCR control were amplified in parallel.

The amplification products were visualized by electrophoresis using 1% weight/volume agarose gel. This was prepared by weighing 1gm of Agarose powder (Agarose low EEO, CSL-AG500, Cleaver Scientific Ltd.) dissolved in 100 ml 1X TBE buffer. The PCR products of mt- HVR were variable in size according to specific species (~550bp to ~850bp). The size of all products was estimated by comparison with 100bp DNA ladder (Thermo Scientific EU).

Table 1: A summary of sequences and product size for all primers used in this research [15].

Primer Name*	Sequence 5'-3	Size
Mammal Primer mt-U1 mt-U2	mt-U1: CCA CCA TCA GCA CCC AAA GCT (21 bases) mt-U2: TGG CCC TGA AGT AAG AAC CAG (21 bases)	Cow 550bp Sheep 850bp
Birds Primer mt–Bd6F mt–Bd7F mt–Bd4R	mt–Bd7F: AGA CCT ACG GCT CGA AAA GCC (21bases) mt–Bd4R: GAT GTG CCT GAC CGA GGA AC (20 bases)	Chicken 600bp

bp: base pair

Table 2: PCR conditions (35 cycles) which used for amplifying HV-regions of mt-DNA [15].

Phase	Mammal (Sheep and Cow)		Birds (Chicken)	
	Temperature (°C)	Duration	Temperature (°C)	Duration
Initialization	98	30 sec	98	30 sec
Denaturation	98	10 sec	98	10 sec
Annealing	60	30 sec	56	30 sec
Extension	72	1 min	72	1 min
Final extension	72	7 min	72	7 min
Holding stage	4		4	

Sec: second, min: minutes, °C: degree centigrade.

Sequencing and database research

DNA sequencing from PCR products and gel electrophoresis was performed using a BigDye® Direct Cycle Sequencing Kit, (cat.no:4458687,100 reactions) as recommended by manufacturer. After the sequencing analysis, the obtained nucleotide sequences were subjected to a BLAST search program (http://www.ddbj.nig.ac.jp/search/blast-j.html) for comparison with the numerous animal mt-DNA sequences on the GenBank sequences database.

Results

More than thirty commercial samples were used in this study. Twenty mammals (10 cows -10 sheep), ten birds (chickens) in addition to the fresh meat control samples were used for genomic DNA extraction. The hypervariable region of the D-loop was amplified by PCR using suitable primers for mammal and birds. The PCR amplified product sizes were identified using agarose gel and by comparison with a 100bp DNA ladder (Figure 1). The difference in product size of the mt-DNA hypervariable region (of D-loop) between different species helped us to identify the species. In case of equal size bands or appearance of extra bands direct sequencing was used to identify the origin of this band.

Identification of type of meat obtained from products known to be made from cow's meat

The PCR amplified mt-DNA hyper variable region (of D-loop) products obtained from fresh, canned and frozen cow's meat products gave the correct specific size of **cow** species (550bp). No extra bands were detected in the gel (Figure 2). For second conformational step, direct DNA sequencing of the obtained bands was done after purification of these PCR products.

The results of the sequenced fragments submitted to BLAST search program in Gen Bank data base are listed in Table 3. The sequences showed that canned and frozen cow meat in addition to the fresh control samples were identified as *Bos taurus* (cow) by 92%, 96% and 98% according to the highest alignments similarity and significant e-values obtained (Figure 5).

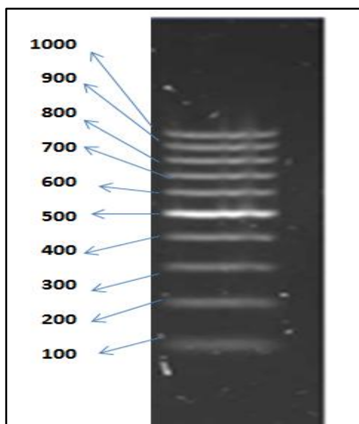
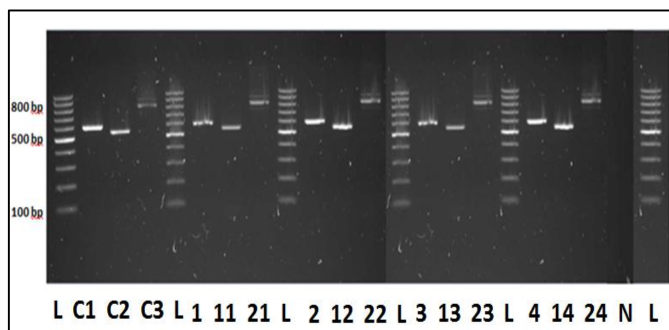


Fig 1: Bands of (100bp) ladder (Nakamura *et al.*2009).



C1 = Chicken control, 1,2,3,4 = Chicken samples, L = Ladder (100bp)
C2 = Cow control, 11,12,13,14 = Cow samples, N = Negative control
C3 = Sheep control, 21,22,23,24 = Sheep samples

Fig 2: Amplification of mt-DNA-HVR for species identification (for cow's, sheep's and chicken's samples (No extra bands were detected in the gel).

Identification of type of meat obtained from products known to be made from sheep's meat

Five PCR amplified mt-DNA hyper variable region (of D-loop) products obtained from fresh, canned and frozen sheep's meat products gave the correct specific size of sheep species (850bp). No extra bands were detected in the gel (Figure 2). For second conformational step, direct DNA sequencing of the obtained bands was done after purification of these PCR products.

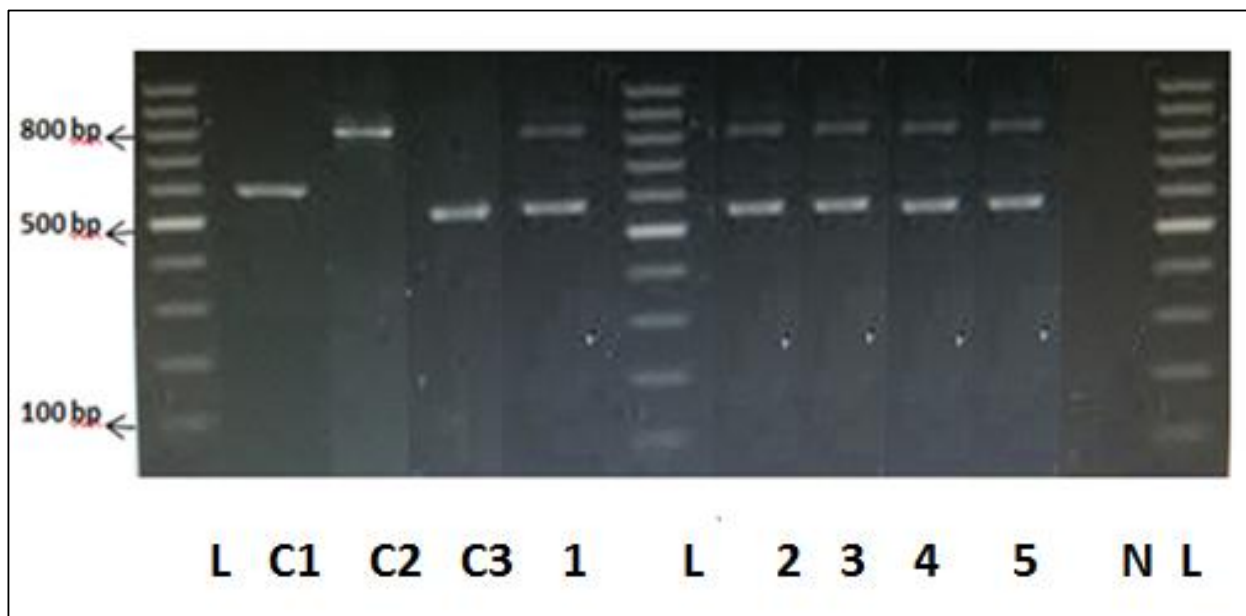
The results of the sequenced fragments submitted to BLAST search program in Gen Bank data base are listed in Table 3. The sequences showed that canned and frozen sheep' meat in addition to the fresh control samples were identified as *Ovis aries* (sheep) by 90% and 96%, respectively according to the highest alignments similarity and significant e-values obtained (Figure 5).

Appearance of extra bands beside the expected normal sheep's bands

The other five sheep samples obtained from products known to be made from sheep's meat showed the normal band 850bp specific for sheep. In addition of these bands extra bands 550bp were also amplified (Figure 3). These extra bands correspond to cow's meat (compared to controls used).

The results of the sequenced fragments (850bp) submitted to BLAST search program in Gen Bank data base are listed in Table 4. The sequences showed that canned and frozen sheep's meat in addition to the fresh control samples were identified as *Ovis aries* and *Ovis arientalis* (sheep) by 85%, 90% and 99%, respectively according to the highest alignments similarity and significant e-values obtained.

The results presented in Table 4 of the sequenced extra bands (550bp) confirmed our observation that the types of meats belong to *Bos taurus* and *Bos indicus* (cow meat) (Figure 4 - 6).



C1 = Chicken control 1, 2, 3, 4, 5 = Sheep samples
C2 = Cow control L = Ladder (100bp)
C3 = Sheep control N = Negative control

Fig 3: Amplification of mt-DNA-HVR show appearance of extra bands beside the expected normal sheep' bands.

Table 3: Identification of cow's, sheep's and chicken's meat using BLAST search results

No. of sample and Common meat name	Species	E. value	Identity of sequence	Accession number
C1 - Control sample (Chicken)	*Gallus qallus*	0.0	99%	DQ-629890.1
(1) Ground chicken	*Gallus qallus*	0.0	98%	KF-800719.1
(2) Minced chicken	*Gallus qallus*	0.0	99%	KC-436031.1
(3) Chicken minced	*Gallus qallus*	0.0	99%	KF-059607.1
C2- Control sample (Cow)	*Bos taurus*	0.0	98%	JN-81735.1.1
(11) Ground beef	*Bos taurus*	4e-167	92%	KF-163074.1
(12) Beef mince	*Bos taurus*	6e-145	96%	KF-163093.1
(13) Beef mince	*Bos taurus*	0.0	96%	AB-973280.1
C3- Control sample (Sheep)	*Ovis aries*	2e-95	96%	KF-938342.1
(21) Minced mutton	*Ovis aries*	0.0	90%	KF-677050.1
(22) Minced mutton	*Ovis aries*	0.0	90%	HM-236175.1
(23) Minced mutton	*Ovis aries*	0.0	90%	KF-938326.1

Table 4: The results of sequences (2 fragments obtained from sheep products) submitted to BLAST search program in Gen Bank data base

No. of sample and Common meat name	Species	E. value	Identity of sequence	Accession number
(26) Mutton mince	*Ovis aries* (sheep) *Bos taurus* (cow)	0.0 0.0	85% 93%	EF-494854.1 JQ-684035.1
(27) Ground mutton	*Ovis aries* (sheep) *Bos taurus* (cow)	0.0 0.0	90% 98%	AY-829409.1 JN-817351.1 (Fig. 4)
(28) Super ground mutton	*Ovis arientalis* (sheep) *Bos indicus* (cow)	1e-107 0.0	99% 90%	KF-938326.1 JX-040461.1

Identification of type of meat obtained from products known to be made from chicken's meat

The PCR amplified mt-DNA hyper variable region (of D-loop) products obtained from fresh, canned and frozen chicken's meat products gave the correct specific size of chicken species (600bp). No extra bands were detected in the gel (Figure 2). For second conformational step, direct DNA sequencing of the obtained bands was done after purification of these PCR products.

The results of the sequenced fragments submitted to BLAST search program in Gen Bank data base are listed in Table 3. The sequences showed that canned and frozen chicken meat in addition to the fresh control samples were identified as *Gallus qallus* (chicken) by 98% and 99% according to the highest alignments similarity and significant e-values obtained (Figure 5).

Sample 27—R41 D2705 14 C10C (sequence code)

Identified nucleotide sequence of the extra band detected in sample No. 27 (Sheep Ground mutton):

```
ATACTATTCCTGACACTATTAATATAGTTCCATAAATACAAAGAGCCTTATCAGTATTAAAT
TTATCAAAAATCCCAATAACTCAACACAGAATTTGCACCCTAACCAAATATTACAAACACCA
CTAGCTAACATAACACGCCCATACACAGACCACAGAATGAATTACCTACGCAAGGGGTAATG
TACATAACATTAATGTAATAAAGACATAATATGTATATAGTACATTAAATTATATRCCCCAT
GCATATAAGCAAGTACATGACYTCTATRGCAGTACATAATACATATAATTATTGACTGTACA
TAGTACATTATGTCAAATTCATYCTTGATAGTATATCTATTATATATTCCTTACCATTAGAT
CACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTAGGCAGGGATCCCTCTTCTC
GCTCCGGGCCCATAAACCGTGGGGGTCGCTATCCAATGAACTTTACCAGGCATCTGGTTCTC
TTTYYCAGGGCCAGTA
```

Alignment with GenBank data base

Bos taurus isolate Mcg489 mitochondrion, complete genome

Sequence ID: gb|JN817351.1|Length: 16339

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
891 bits (482)	0.0	499/509(98%)	3/509(0%)	Plus/Plus	---------

```
Query  7      ACTATT-CCTG-ACACTATTAATATAGTTCCATAAATACAAAGAGCCTTATCAGTATTAA  64
              |||||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15782  ACTATTCCCTGAACACTATTAATATAGTTCCATAAATACAAAGAGCCTTATCAGTATTAA  15841

Query  65     ATTTATCAAAAATCCCAATAACTCAACACAGAATTTGCACCCTAACCAAATATTACAAAC  124
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15842  ATTTATCAAAAATCCCAATAACTCAACACAGAATTTGCACCCTAACCAAATATTACAAAC  15901

Query  125    ACCACTAGCTAACATAACACGCCCATACACAGACCACAGAATGAATTACCTACGCAAGGG  184
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15902  ACCACTAGCTAACATAACACGCCCATACACAGACCACAGAATGAATTACCTACGCAAGGG  15961

Query  185    GTAATGTACATAACATTAATGTAATAAAGACATAATATGTATATAGTACATTAAATTATA  244
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15962  GTAATGTACATAACATTAATGTAATAAAGACATAATATGTATATAGTACATTAAATTATA  16021

Query  245    TRCCCCATGCATATAAGCAAGTACATGACYTCTATRGCAGTACATAATACATATAATTAT  304
              | ||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||
Sbjct  16022  TACCCCATGCATATAAGCAAGTACATGACTTCTATAGCAGTACATAATACATATAATTAT  16081

Query  305    TGACTGTACATAGTACATTATGTCAAATTCATYCTTGATAGTATATCTATTATATATTCC  364
              |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  16082  TGACTGTACATAGTACATTATGTCAAATTCATCCTTGATAGTATATCTATTATATATTCC  16141

Query  365    TTACCATTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTAGGCAG  424
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16142  TTACCATTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTAGGCAG  16201

Query  425    GGATCCCTCTTCTCGCTCCGGGCCCATAAACCGTGGGGGTCGCTATCCAATGAACTTTAC  484
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16202  GGATCCCTCTTCTCGCTCCGGGCCCATAAACCGTGGGGGTCGCTATCCAATGAACTTTAC  16261

Query  485    CAGGCATCTGGTTCTCTTTYYCAGGGCCA  513
              ||||||||||||||| | |  ||||||||
Sbjct  16262  CAGGCATCTGGTTCTTTCTT-CAGGGCCA  16289
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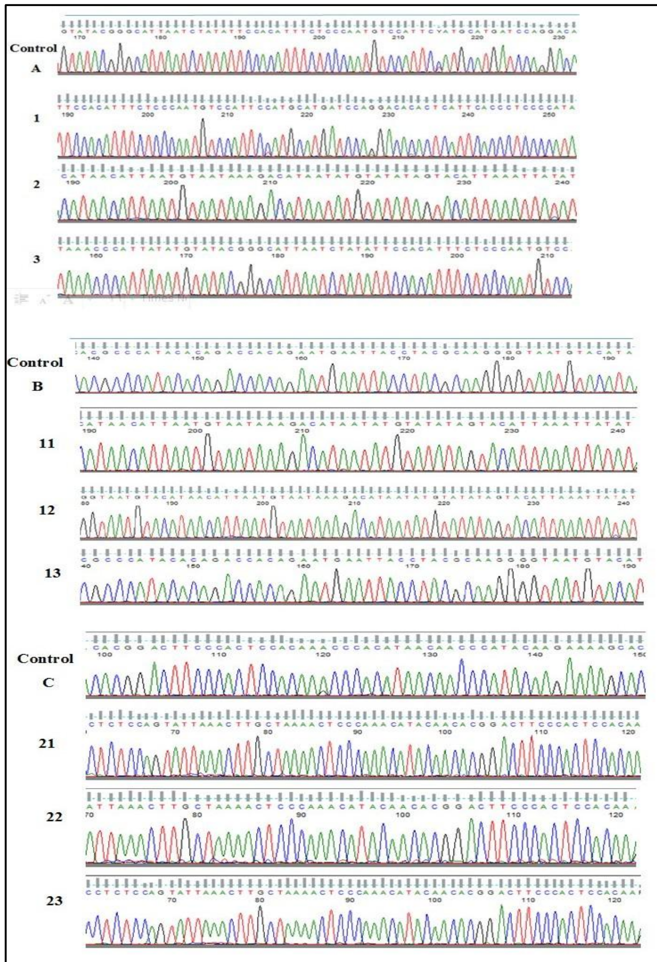
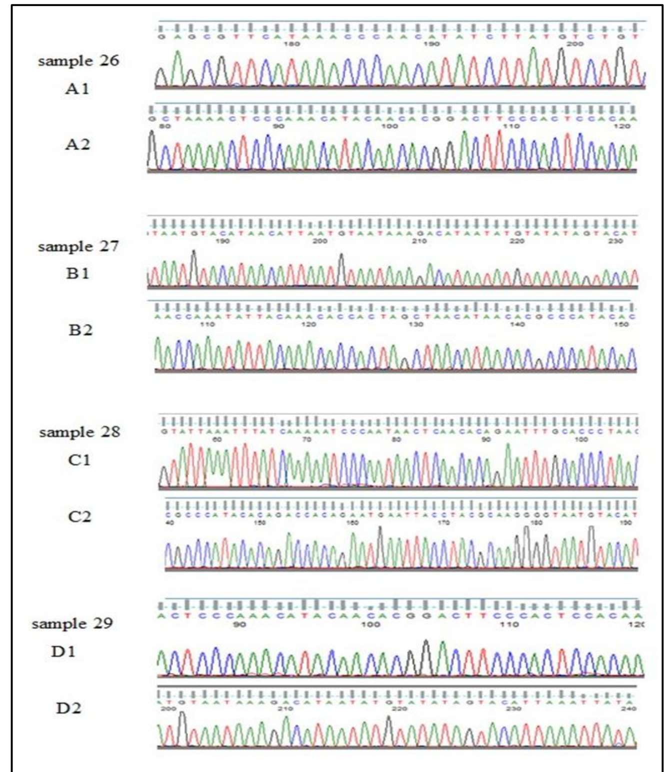
Fig 4: Example of extra band sequencing and identification



Control A = Chicken sample 1,2,3 = Chicken samples
Control B = Cow sample 11,12,13 = Cow samples
Control C = Sheep sample 21,22,23 = Sheep samples

Fig 5: The sequencing of cow's, sheep's and chicken's samples. (No extra bands were detected in the gel)



A1, B1, C1 = Sheep bands
A2, B2, C2 = Cow bands

Fig 6: The sequencing of sheep's samples that show appearance of extra bands beside the expected normal sheep' bands.

Discussion

Different techniques were developed for the identification of the type of meat found in products known to be made from certain species' meat. Identification of certain species were based on electrophoretic techniques [16,7], immunoassays [17,18,19,20] liquid chromatography [21]. These methods were based on the analysis of proteins. These methods showed limitations due to their

complexity, lack of specificity and high cost [22]. The DNA based methods were appeared as a potential tool for identification of species and showed advantages over conventional techniques [23-26].

Mitochondrial DNA sequence is highly conserved in different species of animals. This has enabled designing of universal primers for the HVR of D-loop of mt-DNA, which can amplify corresponding fragments in wide variety of organisms including mammal and birds. General differences between HVR of D-loop of mt-DNA sequences is sufficient for species identification of different biological samples [27].

Molecular techniques, such as polymerase chain reaction [28], randomly amplified polymorphic DNA (RAPD), fingerprinting [19] and gene sequencing [18] have been tried elsewhere for meat identification. Each of these methods has their own limitations. PCR-sequencing is highly repeatable, cheaper and quicker than the methods cites above [4].

Although, DNA sequencing and analysis is accurate and authentic, it is costly, time consuming and not suitable for routine species identification studies. PCR-sequencing has been proven to be a practical, simple and rapid technique [29].

Two main principles were used for species identification by molecular methods. The first method is based on differences in nucleotide sequences (SNPs). This was achieved by amplify DNA fragments from different animals using universal primers. This method requires confirmation of the size of PCR products by gel electrophoresis. Identification of specific (SNPs) was done by nucleotide sequencing of PCR products [6, 8]. This method showed to be tedious but gave exact results.

The second method is based on the differences in the size of PCR fragments produced and were identified by gel electrophoresis [30]. This technique is fast and simple but may sometimes be inaccurate [31]. Both of nuclear DNA and mt-DNA were used for amplification Mitochondrial DNA (mt-DNA) were used as target for amplification for its high copy number and the availability of deposited sequences in Gen Bank database. Universal primers were designed to amplify the D-loop of mt-DNA. However, the fragments obtained were similar in size among all species tested [32].

In the present study, we used a simple system for species identification. It is based on size variations of the amplified mitochondrial DNA hyper variable regions (of the D-loop) among species. Combining DNA size variation and DNA sequencing for the same fragment could be one of the easier and perfect methods for species identification.

Primers used in this study amplified cow, sheep and chicken HVR of D-loop of mt-DNA fragment perfectly. Using universal primers for PCR amplification obviated the requirement for control, which is otherwise used to monitor the success of DNA amplification. As each cell contains about one thousand copies of mitochondrial DNA, PCR assays based on its amplification were shown to be more sensitive as compared to single or low copy nuclear DNA targets. Since, the quantity of PCR products generated corresponds to the copy number of the target DNA sequence, a higher copy number of mitochondrial DNA ensures a sufficiently high quantity of PCR product, even when small amounts of fresh or processed meat samples are used.

The high copy number of small, circular mitochondrial DNA in cells, the chances of their survival under different processing conditions are higher, making it ideal for processed meat species identification. Sequence analysis of HVR of D-loop of mt-DNA showed a significant variation between different species of animals, enabling application of PCR to distinguish them [27].

In a similar report, Nakamura *et al*. (2009) [15] amplified the 550bp fragment of HVR of D-loop of mt-DNA in cow, 850bp fragment of HVR of D-loop of mt-DNA in sheep and 600bp fragment of HVR of D-loop of mt-DNA in chicken, using PCR followed by direct sequence for these fragments.

PCR of HVR of D-loop of mt-DNA could differentiate closely related meat species. It can be concluded that, these three-commercial species, cow, sheep and chicken can be qualitatively identified and differentiated by PCR of HVR of D-loop of mt-DNA. This method can be applied with equal efficiency to both fresh and processed meats [27, 4].

Conclusion

A simple system was used in this study for species identification. The idea based on size variations of the amplified mitochondrial DNA hyper variable regions (of the D-loop) among species. For the confirmation or identification steps there was a need for Combining DNA size variation with DNA sequencing for the same fragment. Size variation could be adequate technique dealing with large number of animal samples without need for more confirmation methods. Sequencing is needed (only with few samples) when there is similarity in bans size or appearance of extra bands. Mitochondrial DNA (mt-DNA) was used as target for amplification for its high copy number and the availability of deposited sequences in GenBank database.

Recommendation

This method of identification of type of species present in meat products showed an easier and un-expansive technique. This technique could be supplementary to other techniques used to protect consumers from commercial fraud by identification of components of introduced or local meat products. This issue is very important for many points of view including, finances, health, religion and detection of adulteration**.**

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