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ORIGINAL ARTICLE

Rapid and Easy Identification of Animal Species for Forensic Application Using New Markers Based on the Mitochondrial 16S rRNA gene

Haider N,[1] Achkar WAL,[2] Nabulsi I.[3]

Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS) , PO Box 6091, Damascus, Syria.

Abstract :

The availability of genetic markers for identification of humans and some animal species by analysis of DNA of these species biological traces that can be found in crime scenes is of great importance as non-deterministic evidence in criminal investigations. Mitochondrial DNA (mtDNA) provides forensic scientists with a valuable tool to determine the source of DNA extracted from damaged, degraded or very small biological samples. The aim of this work was to develop a new, simple, fast and economical method based on polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP, using Tru 1I, Hae III, Taq I, and Dra I) of the mitochondrial 16S rRNA gene (500 bp) for differentiation of human blood form the blood of 11 animals (camel, cow, sheep, goat, buffalo, pig, deer, horse, donkey, dog and cat) and for distinction of the bloods of these animals from each other. The method applied here proved suitable for the differentiation among the analyzed species.

Keywords : Animal species; Forensic; Identification; Mitochondrial 16S; rRNA.

Introduction :

Crime investigators have a unique opportunity to survey the crime scene and identify potential evidence because they are among the first to arrive at the crime scene. The observed spots on the crime scene may be compatible with biological fluids such as saliva, sperm, urine or blood, and may therefore contain DNA that can identify the suspect or victim as well as the acquittal of an innocent individual.[1] Biological traces may be considered as evidence after scientific or technical examination, or may be of no value. Since biological evidence can help resolve the issue, it is necessary to collect and maintain biological samples appropriately.

DNA-based techniques, which emerged in the last decades of the 20th century, are advanced methods by which humans can understand many of the variables around them, and hence DNA became the primary means for investigating crimes. Therefore, the experts of these methods assert that no matter how cautious the offender is, he/she will leave a trace of his/her body in which DNA can be examined.[2] As the DNA technology advances, the time required to test DNA has been reduced from days to hours, making it possible to reduce the process of forensic investigation and judgment.

The genetic fingerprint that is based on DNA analysis is one of the best methods that are currently universally used to identify criminals and uncover the secrets of crimes. The great progress in these methods has made the genetic fingerprint one of the most accurate technical evidence to arrive at a definite or at least certain result, which can be relied upon as conclusive evidence. Consequently, the physical biological traces of crimes and accident scenes have become the traces of strong and conclusive technical evidence on which the judiciary can determine the culprit by examining those traces and comparing the genetic fingerprint with the suspects. Modern and sophisticated DNA analysis techniques have enabled the ability to examine very small and mixed samples accurately. It should be noted here that the genetic fingerprint is identical in all cells of the body (blood, hair, skin, bones, saliva, sweat, urine, mucus, etc.) of a person.

After extracting or isolating DNA from biological material samples for use in the investigation and identification of the offender, there are different methods that may be used for analysis. Analysis of restriction fragment length polymorphism (RFLP) can be considered as the first generation of DNA analysis methods. It is not used at present because it requires large amounts of DNA, and it does not allow analysis of degraded samples accurately. The technology approach was then based on polymerase chain reaction (PCR) and PCR-RFLP techniques. Recent DNA analysis methods include the analysis of short tandem repeat (STR), single nucleotide polymorphism (SNP), mitochondrial DNA analysis (mtDNA) and DNA methylation studies.[3]

The STR[4] is a microsatellite consisting of regions of DNA containing repeated units of 2-6 bp in length that are repeated hundreds of times on the DNA and surrounding the chromosomes centromere. STR's analysis is a tool in forensic science applications for measurement of the number of repeat units. It represents the most frequently used form of genetic information in forensic identification of human biological traces. However, in cases where DNA is highly degraded when exposed to environmental injurious agents or inhibitors, standard STR testing may reveal inadequate. This method also requires a high

Corresponding Author
Nadia Haider
Email : ascientific@aec.org.sy.
Telephone No. : Tel. 00963-11-2132581, 2, 3

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technique and is somewhat difficult.[3] The use of the same technique in animal DNA testing has been largely confined to parental confirmation and genetic testing.[5]

Although most biologists have not care until recently about mtDNA, profiling of this DNA has become routine in forensic biology since mid-1980 and it is regarded as the last resort to test the highly degraded biological traces.[1] MtDNA attracts the attention of forensic workers for many reasons. The size of mtDNA is relatively small compared to nucleic acid, which is more stable because of its circular form,[6] and it has a large number of copies in the cell (500-2000 copies), which increases the likelihood that some copies will remain intact if samples were exposed to vandalization, and therefore, it can be obtained from very small and very old specimens or any samples that do not generate results. MtDNA is inherited from the mother only so it can be used for identification through the use of human remains, the most important of which are blood spots. Amplification of regions of mtDNA is easier in comparison to nuclear DNA (nDNA) due to the greater availability in human and animal cells. Forensic scientists use MtDNA analysis when samples such as bones, teeth and hair are collected from disaster areas or crime scenes. MtDNA is also used when DNA is present in very low or highly dissociated quantities and does not provide a complete picture of STRs.[7] Although it is not unique to the individual, it helps identify or exclude suspects and assist with the progress of the investigation.[6]

Human mtDNA consists of a circular piece of DNA that is 16569 base pairs (bp) and contains 37 genes. In addition to the regions that code for the genes, there is a region that does not represent any gene called the control region. In most animal species, mtDNA also contains 37 genes.[8]

Recently, animal DNA profiling has become more common as researchers are beginning to realize that the same techniques used in human DNA analysis can be also applied to animal evidence

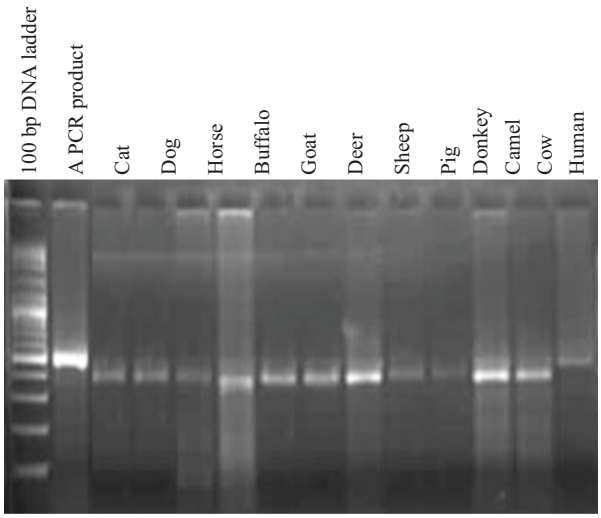

Figure 1. Patterns of restriction products of 16S-Hae IIII as visualized on agarose gel.

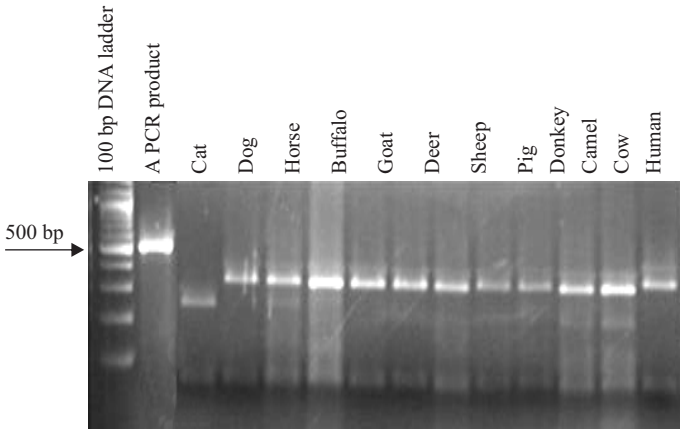

Figure 2. Patterns of restriction products of 16S-Taq I as visualized on agarose gel.

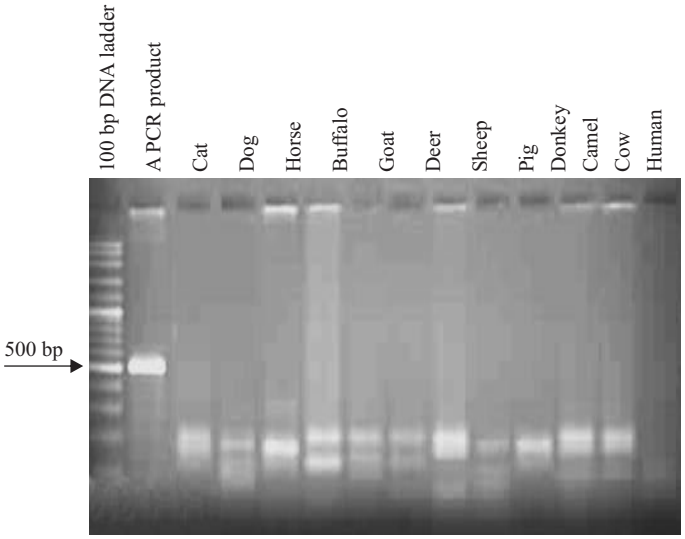

Figure 3. Patterns of restriction products of 16S-Tru 1I as visualized on agarose gel.

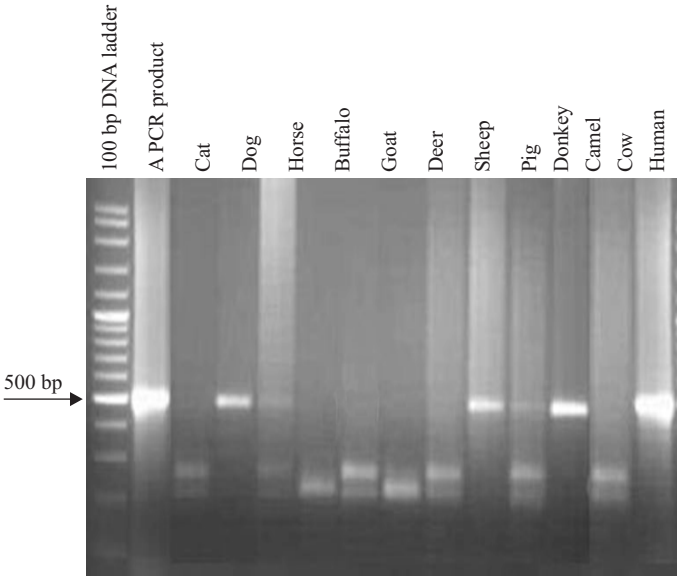

Figure 4. Patterns of restriction products of 16S- Dra I as visualized on agarose gel.

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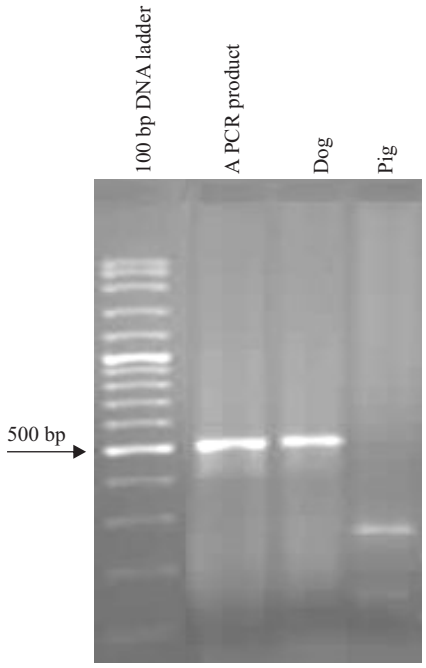

Figure 5. Patterns of restriction products of 16S- Rsa I in dog and pig as visualized on agarose gel.

such as saliva, urine, faeces, blood and hair.[6] Today animal forensics is an important tool, where researchers continue to communicate in cases that are difficult to solve in order to confirm the validity of the investigation. While it is still not widely accepted as human DNA profiling, the characterization of animals DNA has been used in every state of the United States to link both victims of humans and animals with the perpetrators. Animal evidence can also help resolve a wide range of cases of animal cruelty or theft, animal attacks on humans or other animals, and human crimes against humans such as theft, rape and murder.[5]

Minarovič et al.[9] confirmed that PCR on mtDNA is a very efficient method for determining animal species. For example, Nakaki et al.[10] developed a quick and easy method to distinguish between different animal samples (humans, dogs and cats) based on differences in cytokromeb (cyt b) detected by PCR. Morley et al.[11] also developed another method to differentiate between animal species based on mtDNA sequencing. In 2007, Dawnay et al.[12] tested the efficiency of the COI gene sequence for differentiating species at the molecular level for forensic purposes. In a recent study, Natonek-Wiśniewska and Krzyścin[13] explained how mtDNA can help determine the origin of animal species from their traces. The study has shown that mtDNA is suitable for small or highly processed samples the nDNA of which cannot be usually analyzed.

Blood stains are important physical traces because their analysis by DNA techniques may provide us with physical evidence with a great value in proving the presence of the suspect in the crime scene after his/her blood is compared with that recovered from these stains. Therefore, a positive relation between the blood traces and the suspected person may represent a physical

Table 1. A summary of results of PCR-RFLP applied to all studied species.

Species	16S-enzyme combinations/sizes (bp) of bands in restriction products											
	16S	16S	16S		16S-		16S- DdE I			16S- Rsa I		
Cat	420	270	200	18	27	23	She	34	16	Dog	50	
Dog	420	370	180	12	50		Cow	50		Pig	25	13
Horse	420	370	150		27	23						
Buffalo	420	370	200	11	26	23						
Goat	420	370	200	11	27	23						
Deer	420	370	200	11	26	23						
Sheep	420	370	200	18	27	23						
Pig	420	370	180	12	50							
Donkey	420	370	180	12	27	23						
Camel	420	370	200	18	50							
Cow	420	370	200	18	27	23						
Human	500	390	110		50							

evidence of his/her relation to the crime and the absence of such relation excludes this suspect.[2] Profiling of DNA in bloodstains recovered from the crime scene is an important step in daily forensic investigations that are practiced usually at the crime scene and in confirming the origin of the blood whether it belongs to human or not before proceeding with DNA testing.

This work aims to develop a new, simple, fast and economical method based on PCR-RFLP technique applied on a part of the 16S ribosomal RNA (rRNA) gene in mtDNA to distinguish human blood from the blood of eleven animals (camel, cow, sheep, goat, buffalo, pig, deer, horse, donkey, dog, and cat), and to differentiate the animal blood samples targeted here from each other. This method is useful in the field of forensic medicine where it can be used for the determination and distinction of blood samples of these animals easily and accurately.

Materials and Methods :

Sampling : Blood samples were obtained from humans (to distinguish them from the studied animals) and eleven domesticated animals (camel, cattle, sheep, goats, buffalo, pig, deer, horse, donkey, dog and cat).

Isolation of DNA from blood samples : DNA was isolated from fresh blood samples by silica resin method.[14-16]

PCR : A part of the 16S gene of rRNA in mtDNA was amplified in DNAs extracted from the fresh blood samples of targeted human and animals. A pair of universal primers developed by Yang[17] was used to amplify the target region of DNA. The sequences of nucleotides in these primers are as follows:

Forward primer: 5` ACCGTGCAAAGGTAGCATAAT 3`

Reverse primer: 5` TCCGGTCTGAACTCAGATCAC 3`

The composition of PCR reactions was optimized. The final volume was 20 µL including: PCR buffer [Tris-HCl 100mM (pH 8.8 at 25 °C) (NH4) 2SO4 50 mM, Bovin Bromine Serum 0.005% Tween 20 0.00002%], 2.5 mM (MgSO4), dNTPs [0.125 mM for each of the nucleotides (dCTP, dGTP, dTTP and dATP, Fermentas)], 1 unit of Taq DNA Polymerase (Fermentas manufacture), 30-50 ng of DNA and 0.75 mM of each primer.

The amplification program using Eppendorf machine was as follows: - Initial denaturation of the DNA at 94°C for 2 min followed by 30 cycles, each of which included: 1) denaturation at 94°C for 30 sec, 2) annealing of primers at 61°C for 1 min, 3) extension at 72°C for 1 min. Final extension for one cycle at 72 °C for 10 min was also carried out. The reaction products were kept at 4°C until removal from the PCR machine.

PCR products (5 L each) were then separated by electrophoresis on 1.8% agarose gel (Q-BIOgene) that was run at 100 V for 2 h in 1x TAE buffer and visualized under UV lights using Gel Documentation System (GDS8000, UVP). A 100 bp DNA ladder (Vivantis) was used to indicate the positions and sizes of generated bands.

PCR-RFLP : The PCR-RFLP technique was applied on the target DNA region in human and all targeted animal species. This technique is based on the restriction of PCR products using restriction enzymes. To verify the specificity of the restriction pattern to each of the species used and in order to test the efficiency of PCR-RFLP technique in differentiating between these species, it is typically applied to a number of DNA samples representing each of these species, where restriction pattern specific to each species should not vary within species.

Amplification products of the targeted DNA region generated from species analyzed were subjected to restriction digestion with Dra I, Tru 1I, Hae III, and Taq I, where 2 L of the buffer of the enzyme used is added to 0.25 L of the enzyme, 7.75 L of distilled water and 10 L of the amplification product for each DNA sample studied. All restriction reactions were incubated at 37 °C for all enzymes except Taq I (65 °C) for 3 h. An amplification product prior to restriction (for comparison), restriction products, and a 100 bp DNA ladder were separated on 2% agarose gel. In order to distinguish cow from sheep, which could not be distinguished from each other using the four enzymes, Hind II was used only on the amplification products of the target DNA region in these two samples. Dde I was also used to amplify the target DNA region only in donkey and horse to distinguish the latter from each other.

Detection of the specificity of the DNA marker developed for each species studied : The target DNA region (16S) was amplified in the DNA samples extracted from each of the studied species. The number of samples ranged from 3 to 9 (human: 3; camel: 9; sheep and buffalo: 4 each; calf: 6; pig, donkey, goat, dog and cat: 3 each; deer and horse : 1 each). Amplification products of each species were restricted with restriction enzymes that aided its identification.

Results :

PCR : DNAs of good quality were obtained from bloods of all species targeted. This enabled us to amplify the target DNA region in all of these species. When PCR products were visualized on agarose gel, all studied species produced a single DNA band with identical size (500 bp[17]) Compared by the naked eye to DNA ladder.

PCR-RFLP : Table 1 summarizes PCR-RFLP results on the 16S using the four different enzymes (Figures 1-6) applied to all studied species. Markers developed were able to distinguish the blood sample of human from those of animal species. It was also possible to generate PCR-RFLP markers that allowed the distinction of samples of animal bloods from each other.

Detection of the specificity of the PCR-RFLP markers to targeted species : When the specificity of the PCR-RFLP markers to species targeted in this study was detected, no variation was observed in the amplification nor in the restriction patterns among the samples representing each of the species studied.

Discussion :

The criminal investigator relies on the biological evidence that are present in the crime scene. The various biological evidence (blood, flesh, skin, etc.) can tell us 1) the origin of the species to which this evidence belongs, which is very important in forensic science and 2) to distinguish human evidence from animal evidence and 3) if these evidence are of animal origin, which animal they belong to.[18]

In addition to humans, genetic markers are also available in the DNA of some animal species genomes through the analysis of the DNA in these animals` blood cell samples that may be accidentally present at the scene of the crime. These animal markers are of great importance as non-deterministic evidence in crime investigations for their exclusion from human evidence (forensic medicine). Besides being a "witness" at the crime scene, the animals themselves are often victims of crime. Dogs are everywhere in human environments, and they are the most common animals in the crime and forensic sciences, either as evidence or as perpetrators of crime.[19] In addition to the murders of dogs, there is cruelty to animals. Many of the killers have admitted that they started their professional life on animals where animals are often tortured or killed by someone trying to control someone else in cases of domestic violence.

Blood is one of the most biological traces that can be found in crime scenes. Identification of the blood sample and determination whether it is of human or nonhuman origin is one of the most important issues in forensic investigations. When formally identified, the blood sample must be collected and stored appropriately to maintain its integrity at its best for further genetic analysis. Factors that can lead to DNA degradation include time, temperature, humidity, ultraviolet light and exposure to various chemicals.[3] After evidencing a bloodstain, specific techniques are performed to proof the presence of human blood. The main technique according to Advenier et al.[3] is based on chemical analysis that, in contact with blood, induce fluorescence or chemiluminescence or change color.

DNA-based methods are the best option for identifying species that do not have recognizable morphological characteristics. The strength of DNA lies in the fact that although it can be partially disintegrated, its analysis remains possible for months or even years.[3] DNA fingerprinting techniques have been introduced into criminal laboratories to analyze the biological traces associated with crimes, to distinguish between individuals and thus to identify the offender, because the DNA fingerprint is unique and recurs only in one person every few billions of people. These techniques depend on the extraction of DNA from the biological material traces brought from the scene of the crime or from the

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offender or victim, such as blood, semen, saliva, hair, tissues and others. Using these techniques, all biological traces that are very old or very small and degraded can be examined. This is an important advantage since most of the samples associated with the crimes may be degraded or very small.[2]

The mtDNA, as it is the case for the nDNA, varies between species, and thus can be used for legitimate identification. MtDNA profiling is currently used in human forensic analyzes in a variety of situations, especially when DNA is small or fragmented.[19] It may be successfully employed to provide significant progress in the investigation and prosecution of cases with limited biological evidence, such as bones, hair, and skeletal remains,[1] and when cells are dead in the hair stem, as well as in bones and teeth that are thousands of years old.[20] MtDNA is also useful for identification of the remains of soldiers lost in battles, as well as long-time ago deaths, and for determination of whether the perpetrator is actually guilty.[21]

Simplicity, ease of application, accuracy, speed, and low cost to identify a blood sample are important in forensics. PCR-RFLP, which is an essential component of DNA fingerprinting,[22] is a classic forensic technique in murder cases. El-Sayed et al.[23] believe that PCR-RFLP can be successfully used as a routine method of forensic medicine for sensitive, rapid, simple and inexpensive identification of the origin of species in blood spots. The most important advantages of PCR-RFLP are the accuracy, low cost and unnecessity of advanced technologies. Using PCR-RFLP, Bravi et al.[24] identified cattle, horses, donkeys, pigs, sheep, dogs, cats, rabbits, chickens and humans through various restriction patterns of AluI, HaeIII and Hinf I enzymes on cyt b (358 bp). In 2008, Jung et al.[25] concluded that PCR-RFLP is a fast, reliable and simple method that has enabled researchers to identify six species of fish (cyt b, 465 bp). In another study, Nakaki et al.[26] compared the PCR-RFLP pattern of the *cyt b* (bp 700) to identify animal species for forensic science studies using total DNA isolated from human blood and eight mammal species (baboon, cow, pig, dog, cat, bear, deer, and raccoon dog) and two birds (chicken and ducks). However, it was not possible to amplify the targeted DNA in some species. The authors attributed this to the mismatch of the primers used with the targeted DNA portion. In a recent study, Farag et al.[27] revealed that the PCR-RFLP analysis provides a fast and effective way to detect the origin of species.

The aim of this study was to evaluate for the first time the use of genetic markers generated from analysis of 16S of mtDNA in bloods of human and some animals for forensic purposes using PCR-RFLP. Blood samples taken from humans and eleven animals (camel, cow, sheep, goat, buffalo, pig, deer, horse, donkey, dog, and cat) were studied. The variation detected in 16S could be used as a useful tool to determine the identity or origin of the targeted species for forensic purposes, because it was possible to identify the human blood sample from the bloods of all animals analyzed here using one enzyme Hae III, which restricted in all animals but did not restrict in human blood. The polymorphism revealed in 16S using three additional enzymes also allowed identification of all animals studied apart from sheep from cow and dog from pig. Using two additional enzymes, we were able to distinguish dog from pig and sheep from cow. This method has shown great potential for distinction of animal species even those which are closely related.

Analysis of DNA of animal samples for identification purposes at the species level usually includes sequencing of whole or a portion of mtDNA (DNA barcoding, see Haider et al.[28] which is then compared to known Genebank sequences (National Center for Biotechnology Information, NCBI). The DNA barcoding method has recently been adopted as a fast and accurate global tool for species identification.[29] These barcodes have proven highly effective in distinguishing many animal groups[30] using DNA sequencing. Although DNA sequencing analysis is accurate and reliable, it is costly, time and work-intensive, and the samples may not produce sufficient or useful sequence data.[31] There are other molecular techniques that allow the identification of markers specific to species such as mtDNA-RFLP, PCR-RAPD and SSRs.[27] Of these, mtDNA-RFLP analysis has significant advantages that make it superior to other analyses including sequencing. PCR-RFLP requires less equipment than DNA sequencing, resulting in simple data that is easy to analyze and compare. PCR-RFLP has been revealed here to be a reliable and reproducible method such as that of DNA sequencing, but it is cheaper, faster, easier and can be applied in any biotechnology laboratory. Therefore, it can be used as an effective and inexpensive alternative to direct DNA sequencing to distinguish human blood from the bloods of the animals we studied, as well as to distinguish the blood of the latter from one another in forensic fields, even if the DNA is little or degraded when DNA is extracted from old or dry blood samples as proved by Haider and Jarjour (unpublished data), who applied successfully PCR-RFLP on mtDNA using total genomic DNA they extracted, following the protocol of Qiagen Miniprep kit (cat No. 51304), from dried blood samples (blood spots on pieces of cloth and filter paper) of humans and some animals such as cow, sheep, camel, mouse, chicken, lion, baboon, dog and wildcat.

Because the targeted 16S region of the mitochondrial genome is small (500 bp), it can be amplified even if the target DNA samples are old and degraded in one PCR experiment and restriction with 1-4 restriction enzymes. Species-specific markers developed here can be used to quickly, accurately, and easily distinguish the blood spots found in a crime scene or in any humans or animals-related investigations. And because mtDNA is uniform in all living cells of humans as well as in the same animal species, these markers can be useful in determination of other biological traces (hair, tissue, bone, saliva, etc.).

Although the sequences of primers used here to amplify the target portion of the 16S gene were previously published,[17] this is the first attempt to analyze this region of mtDNA and to detect its variations (mutations) to develop simple and reliable PCR-RFLP markers specific to humans and to each of the animals studied. These markers are useful for forensic purposes to analyze blood traces (or other biological traces) that may exist in crime scenes or accidents involving humans or one or more of the targeted animals.

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