Mitochondrial DNA Quantification in Animal Blood and Hair by Slot-Blotting

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INTRODUCTION

The hairs of cats and dogs easily cling to various objects whose surfaces are not entirely smooth; the result is that hairs are constantly found on clothes, furnitures, carpets, etc. which are in contact with the everyday life of these animals. This type of evidence could become an extremely important evidential value in the investigations of certain crimes and offences. By their interesting unusual presence in certain places, they may give interesting informations on the circumstances of the crime. Hairs are potential sources of DNA. However, the samples which are analysed often have roots of bad quality or even no roots at all, this means that there is a great risk of not having enough genomic DNA to carry out an analysis, even by PCR. It is therefore interesting to analyse the mitochondrial DNA (mtDNA) of the shaft of the hair. As for the analysis of genomic DNA, to know the amount of DNA available would be a precious information.
A quantification of mitochondrial DNA by slot-blotting has therefore been attempted by using oligonucleotide probes coupled to alkaline phosphatase.

MATERIALS AND METHODS

Blood: total DNA was extracted from cat, dog and human blood by using the phenol/chloroform method: extraction overnight at 37°C in a solution of 10mM Tris-HCl pH7.5, 10mM EDTA pH7.5, 100mM NaCl, 2% SDS, containing 6mM DTT and 1.5mg/ml proteinase K. DNA was then precipitated with alcohol in presence of 0.022mg/ml glycogen and 2M NH_4Ac and dissolved in water (Piercy et al., 1993).

Hair: 50 cat hairs per sample were used for the extraction of the total DNA, roots and shafts being separated. The same procedure was carried out for dog hair. A purification process using Microcon™ 30 microconcentrators (Amicon, Beverly, MA, USA) has been attempted on a few samples.
The most coarse hair, having roots of average to good quality, were taken by stroking the animal.
Before extraction, shafts were washed in sterile water. Both roots and shafts were extracted overnight at 56°C in a solution of 50mM Tris-HCl pH8, 10mM EDTA pH8, 0.1M NaCl, 2% SDS, 0.2M DTT and proteinase K 3.2mg/ml (for the roots) and 0.27mg/ml (for the shafts). DNA was then precipitated in the same way as abovementioned.

Preparation of pure mtDNA: mtDNA was isolated after extraction of a cats liver, respectively of a dogs liver by following the procedure described by White and al. (1992) uses differential centrifugation of the mitochondria and DNA purification by salt-precipitation of the proteins followed by an organic extraction (White *et al.*, 1992).

DNA quantification: The mitochondrial DNA quantification was attempted by slot-blot hybridization using a nylon membrane in a non-radioactive format. The procedure was similar to the "Human Quantification System" (Gibco-BRL, Gaitherburg, MD, USA). The detection buffer was substituted by a solution of 0.1M diethanolamine, 1mM magnesium chloride pH9.5 and the chemiluminescent substrate used was CDP-Star™ (Tropix, Bedford, USA). The totality of each extract containing the 50 shafts, respectively the 50 roots were deposited on the membrane.

Probes: 4 cat probes and 3 dog probes were tested at different temperatures:

Probe name	*Sequence*	*Hybridization temperatures tested*
(Cat) B17	5'- AAT CAC ACC CCC TTA TCA -3'	52; 50; 44°C
(Cat) F207	5'- CTG TCG CGA CGT TAA TT -3'	50; 44°C
(Cat) F385	5'- ATG GGA TAC GTC CT -3'	50; 44; 33°C
(Cat) F241	5'- TAT TTA CAC GCC AAC GGA GC -3'	52; 50; 46°C
(Dog) C230	5'- GAA TTA TCC GCT ATA T -3'	50; 46; 40°C
(Dog) C243	5'- TAT GCA CGC AAA TGG CGC -3'	54; 50; 45°C
(Dog) B291	5'- TGT AGG ACG AGG CCT ATA-3'	50; 48; 45°C

The sequences belong to the cytochrom b of the mtDNA of these animals. They were chosen after consultation in the Genbank (The National Center for Biotechnology Information, Bethesda, Maryland). They were conjugated to an alkaline phosphatase enzyme according to an E-Link™ Oligonucleotide Labelling kit (Cambridge Research Biomedicals Ltd, Cheshire, UK) except for two probes (C230; C243) which were synthetized and conjugated by Genset SA (Paris, France).

Southern blotting: cat and dog DNA extracts were analysed on 0.6% agarose gel followed by staining with ethidium bromide and transferred on a nylon membrane. They were then hybridized with the probes in order to verify the specificity for mtDNA.

RESULTS AND DISCUSSION

The most sensitive and specific probe for cat DNA was found to be the F241 probe at an hybridization temperature of 50°C. Southern blotting demonstrated the specificity of the probe and the absence of cross-hybridization with genomic DNA (fig. 1).
The concentration of the mtDNA isolated from liver was estimated by comparison with DNA marker on a minigel stained with ethidium bromide. This mtDNA extract could then be used as a standard for slot-blotting quantification. It was then possible to estimate the amount of mtDNA in blood and hair (fig. 2) :

Cat samples	*Estimation of the mitochondrial molecules number* [1]
1 µl blood	about 2'000 000
1 hair root	about 200'000
1 hair shaft	about 200'000

[1]As an element of comparison, it is interesting to note that the 200'000 copies of the mitochondrial genome detected in a hair shaft would correspond to 1.2 µg of DNA if this was nuclear DNA instead of mtDNA.

The estimation of the mtDNA amount in hair shafts was difficult because of interference in the detection of the signal. This phenomenon mainly occured while using dark-coloured hair, meaning that melanin might well be the interfering molecule. A purification process (filtration over Microcon™ 30 microconcentrators) was attempted in order to remove this inhibition but no better results were obtained and some DNA was even detected in the filtrate (fig. 2).

The most sensitive and specific probe for dog DNA was found to be the B291 probe at an hybridization temperature of 48°C. The chosen sequence is complementary to a mitochondrial sequence and not genomic but this was not totally confirmed. This probe is less sensitive compared to the cat probe and the same inhibition phenomenon was observed.
The amounts of mtDNA which were detected in blood, in a root and in a shaft of a dogs hair are similar to those determined for the cat.

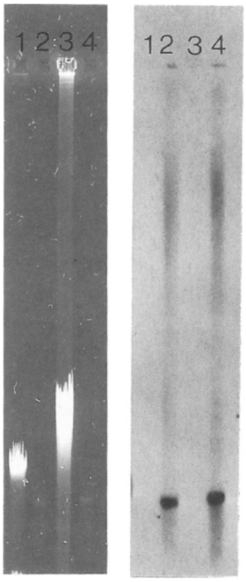

Figure 1. *Left*:: agarose gel stained with ethidium bromide. *Right*:: DNA transferred on a membrane and hybridized with the cat probe F241
1. Hind III Digest of Lambda DNA marker (band 1: 23'130 base pairs)
2. Cat mitochondrial DNA
3.Total DNA extracted from 12 µl of cat blood
4.Cat mitochondrial DNA

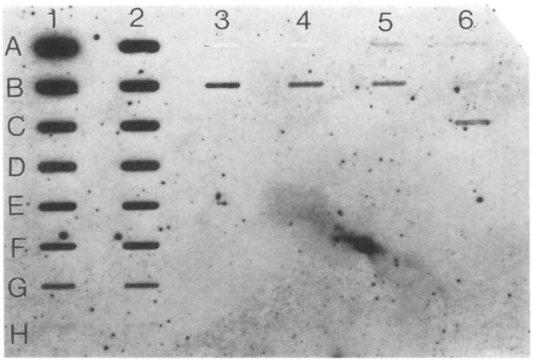

Figure 2. An example of the quantification of mitochondrial DNA present in blood, hair roots and hair shafts of cats.
Column 1: standards (liver extract) containing 1A = 6 ng, 1B = 3 ng,1C = 1 ng, 1D = 0.8 ng, 1E = 0.4 ng, 1F = 0.2 ng, 1G = 0.1 ng, 1H = 0 ng of mitochondrial DNA.
Column 2: Total DNA extracted from 60 µl (2A), 42 µl (2B), 30 µl (2C), 18 µl (2D), 12 µl (2E), 6 µl (2F), 3 µl (2G) of blood.
Samples 3A, 4A, 5A : amount of mtDNA present in 50 hair shafts of three different cats after purification with Microcon™30 microconcentrators.
Samples 3B, 4B, 5B: amount of mtDNA present in each 50 corresponding roots without purification.
Sample 6A: filtrate of sample 3A, 6B: filtrate of sample 4A, 6C: filtrate of sample 5A.

Blood is a relatively rich source of mitochondrial DNA and not surprisingly, the roots contain more mitochondrial DNA molecules than the shafts. According to the estimations, the cats hair, respectively the dogs hair, contain enough mitochondrial DNA to carry out an analysis in the aim of an individual identification by identical methods to those used in human identification.

We were hoping to be able to estimate the amount of mtDNA in hair and to devise a quantification method which might help in routine mtDNA analysis. Regarding to the results, this second goal could not be reached because of insufficient sensitivity. Therefore, in order to quantify mtDNA in single hair, it would then be necessary to look for other methods than slot-blotting.

REFERENCES

Piercy R, Sullivan KM, Benson N, Gill P (1993) The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. Int J Leg Med 106: 85-90
White PS, Densmore LD (1992) Mitochondrial DNA isolation. In: Hoelzel AR (ed) Molecular Genetic analysis of populations: a practical approach. IRL Press, p 29-58.