World Rabbit Science

World Rabbit Sci. 2007, 15: 121 - 126

NON-INVASIVE AND SIMPLE METHODS FOR SAMPLING RABBIT DNA FOR PCR ANALYSIS OF MELANOCORTIN 1 RECEPTOR (MC1R) GENE MUTATIONS: A TECHNICAL NOTE

Fontanesi L., Tazzoli M., Russo V.

Dpt. Protezione e Valorizzazione Agroambientale (DIPROVAL), Sz. Allevamenti Zootecnici, Univ. of Bologna, Reggio Emilia, Italy

Abstract: Simple and non-invasive methods of collecting and preserving biological specimens for genetic analysis can be useful in reducing the discomfort of the involved animals and can be applicable for high-throughput studies that require a large number of biosamples. In order to have the possibility to analyse a large number of samples and making easier the collection of rabbit biological materials as well as to reduce the cost and time of the genotyping procedures, here we tested different protocols for the genotyping of mutations identified in the rabbit melanocortin receptor 1 (MC1R) gene that are associated to coat colours in different rabbit breeds. These protocols make use of rabbit hair roots or cell preparations from buccal swabs with a direct PCR amplification without previous DNA extraction. Hair samples were collected from 60 rabbits of different breeds and preserved for up to 12 months at 4°C. For each hair sample 5-10 hair roots were clipped a few mm from the root directly within a 0.2 ml PCR tube. Buccal cells were collected from 10 crossbred and purebred rabbits and prepared after a few steps for PCR. The PCR fragments were analysed by means of polyacrylamide gel electrophoresis and stained with ethidium bromide or using fluorescent labelled products resolved in a capillary automatic sequencer. The success genotyping rate of the hair and buccal cell samples was 98% and 80%, respectively. These values are comparable to the success rates of other protocols described in human or other animals involving previous DNA extraction. The obtained results show that non-invasive methods of biological specimen collection that make it possible to analyse the DNA without previous extraction can be easily and routinely applied in rabbit molecular genetic studies. However, due to the higher success rate and the easier method of collection, plucked hairs seem the best source for high-throughput biosampling in this species.

Key words: Rabbit, hair DNA, buccal swabs, biological specimens, direct PCR, MC1R mutations.

INTRODUCTION

Usually, peripheral blood sampling has been the method of choice for preparing animal DNA for a wide range of molecular genetic applications and classical protocols use this biological material for DNA extraction (i.e.: Sambrook *et al.*, 1989). However, its collection requires invasive procedures and the use of anticoagulants, like EDTA or epinephrine, complicating sometimes the sampling and storing. Thus, alternative, simple and non-invasive methods of collecting and preserving biological specimens for DNA extraction can be useful in reducing the discomfort of the involved animals as well as to facilitate these procedures.

For these reasons, several sampling approaches including the use of hairs and buccal swabs have been largely and successfully applied in human epidemiological studies and forensic medicine (Higuchi *et al.*, 1988; Thomson *et al.*, 1992; Walker *et al.*, 1999). The use of hair as source of DNA has been

Correspondence: L. Fontanesi, luca.fontanesi@unibo.it
Received *December 2006* - Accepted *February 2007.*

described in dogs and cats (Menotti-Raymond *et al.*, 1997; Oberbauer *et al.*, 2003; Pfeiffer *et al.*, 2004) as well as in wild animals (Sloane *et al.*, 2000; Roon *et al.*, 2003; Frantz *et al.*, 2004) for forensic, genome analysis and population genetic studies. In large farm mammals, like, for example, pigs and cattle, it is common the use of hair roots as source of DNA in PCR studies (Healy *et al.*, 1995; Russo *et al.*, 1996). However, in small mammals, like the rabbits, the use of hair roots has not been described yet. Less frequent in any farm animal is the use of buccal swabs whose collection could be more complicated in large than in small animals (Brooks *et al.*, 2003; Meldgaard *et al.*, 2004).

Then simple PCR protocols accompanying these non-invasive biological specimen collecting methods make it possible to reduce the analysis costs speeding up the laboratory work. A large number of PCR protocols has been described starting from the isolation of DNA using alternative and simple methods. Some of them use directly biological materials, like blood, serum, cerebrospinal fluid, saliva and solid tissues, without any prior DNA extraction step (Mercier *et al.*, 1990; Panaccio *et al.*, 1993a; 1993b; Liu *et al.*, 1995; Sandford and Pare, 1997; French *et al.*, 2002; Wong *et al.*, 2003). To our knowledge, hair roots and buccal cells have not been used in direct PCR experiments, yet. Then, visualization of the amplified DNA can be performed using standard gel electrophoresis with ethidium bromide staining or using capillary electrophoresis of dye labelled fragments in automatic sequencers.

We recently identified two mutations in the rabbit melanocortin 1 receptor (MC1R) gene (the *Extension* locus) associated with red or black coat colours in different rabbit breeds. These mutations were caused by the deletion of 30 or 6 base pairs (bp) of the single exon MC1R gene determining the production of a putative non functional or activated transmembrane receptor, respectively (Fontanesi *et al.*, 2006).

In order to analyse a large number of samples and making easier the collection of rabbit biological materials as well as to reduce the cost and time of the genotyping procedures, here we describe different protocols for the genotyping of mutations identified in the rabbit MC1R gene that make use of rabbit hair roots or cell preparations from buccal swabs with a direct PCR amplification without any preliminary step of DNA extraction.

MATERIALS AND METHODS

Hair and buccal swab sampling.

Hair samples were collected from 60 rabbits of different breeds (Belgian Hare, n=5; Blue Vienna, n=5; Burgundy Fawn, n=10; Californian, n=10; Checkered Giant, n=5; Giant Grey, n=4; New Zealand White, n=20; commercial hybrid, n=1) and preserved for up to 12 months in 1.5 ml polypropylene microtubes at 4°C. Buccal cells were collected from 10 crossbred and purebred rabbits. For all animals the MC1R genotype was previously known based on PCR analyses from extracted DNA samples (Fontanesi *et al.*, 2006). For buccal cell sampling, widely available sterile cotton sticks were used to gently scrape of both inner cheeks of the animals. Then the cotton sticks were cut a few mm from the cotton bud that was dropped in a 1.5 ml microcentrifuge tube. Closed tubes were placed for 2-5 days at 4°C.

Biological material for direct PCR.

With the help of magnification lens and sterile laboratory forceps and scissors, for each hair sample 5-10 hair roots were clipped a few mm from the root directly within a 0.2 ml PCR tube. Then, the tubes were closed and stored at room temperature (RT) till their use for PCR. This procedure was carried out in a clean and sterile environment and care was taken to avoid cross contamination between samples. On average, each plucked hair makes it possible to obtain hair roots for about 10 PCR.

The preparation of the buccal cells was modified from Meldgaard *et al.* (2004). Briefly, about 0.6 ml of sterile bidistilled H_2O was added to the tubes containing the cotton buds prepared as described

above. The cotton buds were allowed to soak for 10 min at RT while the tubes were rotating on a suitable device. Then the tubes were vortexed thoroughly and spinned for 30 sec in a microcentrifuge. The tubes were opened and the cotton buds were removed and disposed after having pressed them against the interior surface of the microcentrifuge tube in order to release as much liquid as possible. After, the tubes containing the remained liquid were centrifugated for 5 min at 5,000 rpm. Water was pipetted off while taking care not to disturb the small cell/debris pellet. Then, 50 µl of PCR buffer 1X was added to the pellet and the tubes were vortexed until the pellet disappeared.

Negative controls were incorporated into all biosample preparations.

PCR and genotyping analyses.

Target of the PCR amplification was a 146 bp fragment (dimension of the wild type alleles) of the rabbit MC1R gene encompassing the region containing the two deletions associated with different coat colours (Fontanesi et al., 2006). The PCR tubes containing the hair roots were filled with 20 µl of PCR mix and then briefly spinned. PCR mix contained 1 U EuroTaq DNA polymerase (EuroClone Ltd., Paington, Devon, UK), 1X PCR Buffer, 2.5 mM dNTPs, 10 pmol of primer forward (5'-ATCTGCTGCCTGGCCTTG-3') and 10 pmol of primer reverse (5'-CAGATGAGCACGTCGATGA-3'), 1.0 mM of $MgCl_2$, bidistilled H_2O to the final volume of 20 µl. The buccal cells were amplified directly using the same PCR mix described above and as template 5 µl of 1X PCR buffer after the suspension of the cell pellet as indicated in the previous paragraph (thus, each buccal cell preparation makes it possible to perform about 10 PCR). As controls, four samples of rabbit DNA extracted from whole blood using the protocol described in Sambrook *et al.* (1989) and negative controls were used in all PCRs.

PCR profile was as follows: 8 min at 95°C; 40 amplification cycles of 30 sec at 95°C, 30 sec at 62°C, 30 sec at 72°C; 10 min at 72°C. PCR was performed using a PT-100 thermal cycler (MJ Research, Watertown, MA, USA). When the amplified fragments were resolved using capillary electrophoresis in an ABI3100 Avant sequencer (Applied Biosystems, Foster City, CA, USA), primer forward was fluorescence labelled at 5min with the dye 6-FAM. For this analysis, PCR products (2 ml reaction product) were diluted in 10 ml of Hi-Di formamide (Applied Biosystems) and added to 0.1 ml of Rox labelled DNA ladders (500HD Rox, Applied Biosystems). Labelled DNA fragments were scored using GeneScan v. 3.7 and Genotyper v. 3.7 software (Applied Biosystems). PCR primers were not labelled when the detection of the DNA fragments was carried out using 10% 29:1 bis-acrylamide:acrylamide gel electrophoresis and ethidum bromide staining.

No amplification was detected in all negative controls.

RESULTS AND DISCUSSION

Direct PCR of biological materials or tissues relies on the possibility to get free DNA in solution after the denaturation steps that should lysate cells. However, the presence of *Taq* polymerase inhibitors may prevent PCR amplification and for this reason special PCR mixes have been applied in direct PCR analyses (Panaccio *et al.*, 1993a; 1993b). Nevertheless, it is possible that biological samples may not require any particular treatment as PCR inhibitors are not abundant or are diluted and do not affect amplification reactions (Fode-Vaughan *et al.*, 2001). Here we tested this possibility in order to routinely continue to carry out PCR without any modification from standard protocols for the analyses of mutations of the rabbit MC1R gene. As the genotyping of the two MC1R deletions is based on fragment length polymorphisms (Fontanesi *et al.*, 2006), no further treatment of what is produced from the amplification reactions is needed to score the rabbit genotypes making it easier to test new protocols for this analysis. Two sources of biological materials containing DNA were tested: i) hair roots stored for up to one year and ii) buccal cells. Sampling hair is an extremely simple way to collect

biological material in rabbits and hundreds of samples can be easily obtained in a short time. Of the 60 analysed samples of the present work we obtained the expected PCR fragment for 54 animals (90% of positive results) in the first attempt of amplification. The six failed samples were then reanalysed taking care to check if the brief spinning of the PCR tubes after the introduction of the PCR mix made it possible to obtain a direct contact of the hair roots with the PCR solution. In the second attempt, for five of them it was possible to obtain the genotype. The reason for which a sample was not possible to amplify was probably due to the low number and/or poor quality of the hair roots selected from a very small pluck of hair. Thus, on the whole, it was possible to genotype ~98% of the samples that is a success rate higher or comparable to what was observed in human or animal forensic and population studies that involved hair DNA extraction protocols (Sloane *et al.*, 2000; Frantz *et al.*, 2004; Pfeiffer *et al.*, 2004; Melton *et al.*, 2005). Figure 1 shows some examples of the amplified fragments resolved with polyacrylamide gel electrophoresis and stained with ethidium bromide. The obtained genotypes were as expected according to what was already reported by Fontanesi *et al.* (2006). Moreover, a protocol using a dye labelled primer in PCR amplification and fragment analysis operated by a capillary sequencer was set up. Figure 2 shows some electropherograms from rabbits with different MC1R genotypes. This method is more sensitive as DNA amplified fragments were correctly scored even if the same amplifications checked on polyacrylamide gels could not be clearly visualized. The genotypes obtained with this method of analysis confirmed the results obtained with polyacrylamide gel electrophoresis analyses.

Then, this protocol was used to test the possibility to detect the genotype at this rabbit locus starting from buccal cell crude preparations without DNA extraction. Of the ten analysed samples, eight gave the expected genotype while for two it was not possible to amplify the DNA even if the amplification step was attempted three times (80% of positive results). Further adjustments to this protocol may be required to obtain a more efficient result. However, it should be considered that this

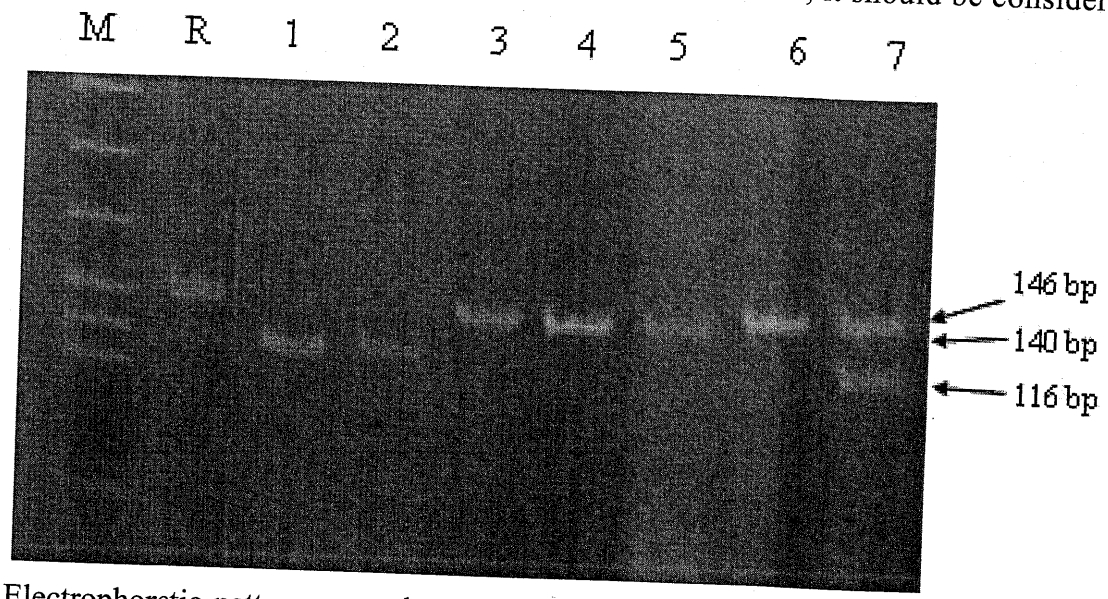

Figure 1. Electrophoretic patterns on polyacrylamide gel of the DNA fragments obtained for the rabbit *MC1R* gene after direct PCR of hair root samples. **M**, DNA molecular weight marker VIII (Roche Molecular Diagnostics, Mannheim, Germany); **R**, reference mix of the amplified products of three alleles (the wild type allele of 146 bp, the 6 bp deletion allele of 140 bp and the 30 bp deletion allele of 116 bp) at the *MC1R* locus (Fontanesi *et al.*, 2006); **1** and **2**, fragments of 116 bp obtained from two Burgundy Fawn rabbits (the animals are homozygous for the 30 bp deletion allele); **3**, **4** and **5**, fragments of 140 bp obtained from three Californian rabbits (the animals are homozygous for the 6 bp deletion allele); **6**, fragment of 146 bp obtained from a Giant Grey rabbit (the animal is homozygous for the wild type allele); **7**, pattern of a heterozygous rabbit (commercial hybrid) carrying the wild type allele and the 30 bp deletion allele.

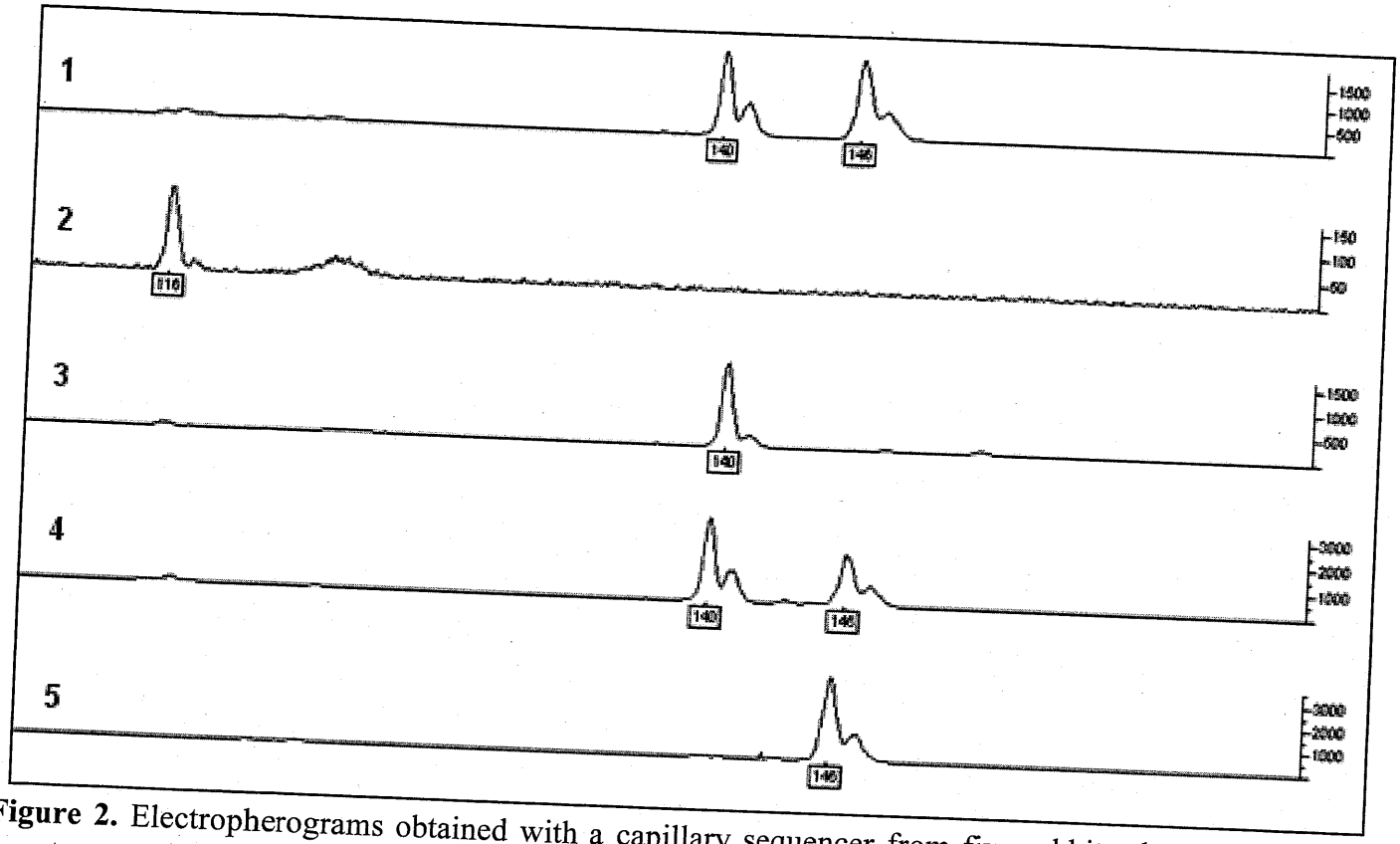

Figure 2. Electropherograms obtained with a capillary sequencer from five rabbits showing different genotypes at the *MC1R* locus. **1** and **4**: heterozygous rabbits for the wild type and the 6 bp deletion alleles; **2**, homozygous rabbit for the 30 bp deletion allele; **3**, homozygous rabbit for the 6 bp allele; **5**, homozygous rabbit for the wild type allele.

method of cell preparation may produce a very low amount of amplifiable DNA due a direct amplification of the cell debris without previous DNA extraction step. Even if this data has been obtained from a few samples, still this can be considered a high success rate comparing our method to the DNA extraction protocols applied in human forensic and epidemiological studies that used buccal swabs in which PCR was successful in ~75% to ~99% of all cases according to the amplified locus or the method of collection of biosamples (Walker *et al.*, 1999; Feigelson *et al.*, 2001).

The obtained results showed that non-invasive methods of biological specimen collection that make it possible to analyse the DNA can be easily applied in rabbit molecular genetic studies. Direct PCR protocols for the analysis of the *MC1R* gene without preliminary DNA extraction have been tested successfully in this species considering hair roots and buccal cells. Thus, it could be reasonably predicted that the described protocols can work also for other PCR products. However, this has not been tested in the present work and needs further investigations with a particular attention to the possibility to amplify mirosatellite loci. As a matter of fact, microsatellite analyses from low quality or low quantity DNA can be affected by two types of genotyping errors: i) allele dropout for which a true heterozygote is scored homozygote and ii) false allele production defined as a PCR-generated allele that is not one of the alleles of the true genotype (Goossens *et al.*, 1998). These problems have not been encountered in the fragment analysis for the MC1R gene whose polymorphic sites are not simple sequence repeats: the obtained genotypes in the investigated animals were always as expected from previous data that were produced by PCR from extracted DNA. Probably, a more in-deep evaluation of the error rate of the described genotyping protocols should include a larger number of samples. Nevertheless, it is still possible to consider, before PCR amplification, a DNA extraction step from the two investigated sources of biological material. In this case, it remains the advantage of the simplicity of the methods of specimen collection that are easier than blood sampling.

On the other hand, comparing the results obtained with the use of two sources of biological materials (hairs or buccal cells), due to a higher success rate and an easier method of collection, plucked hairs seem the best source for high-throughput biosampling in the rabbit. Thus we will continue to use this method for a more in-depth investigation of the rabbit MC1R gene polymorphisms that may include analysis of other breeds/lines, family based pedigrees and wild rabbit populations.

Moreover, it should be pointed out that hair root analyses resulted reliable for the genotyping of samples stored for up to one year. Further investigations to evaluate in more detail the storage time between collection and use of this material is under way.

Acnowledgements: This work was supported by RFO funds from the University of Bologna. We thank several rabbit breeders and Associazione Nazionale Coniglicoltori Italiani (ANCI) for their collaboration in the sampling of biological materials.

REFERENCES

Brooks R., Williamson J., Hensley A., Butler E., Touchton G., Smith E., 2003. Buccal cells as a source of DNA for comparative animal genomic analysis. *Biotechnology Lett.*, 25, 451-454.

Fode-Vaughan K.A., Wimpee C.F., Remsen C.C., Collins M.L., 2001. Detection of bacteria in environmental samples by direct PCR without DNA extraction. *Biotechniques*, 31, 598-604.

Fontanesi L., Tazzoli M., Beretti F., Russo V., 2006. Mutations in the *melanocortin 1 receptor* (*MC1R*) gene are associated with coat colours in the domestic rabbit (*Oryctolagus cuniculus*). *Anim. Genet.*, 37, 489-493.

Frantz A.C., Schaul M., Pope L.C., Fack F., Schley L., Muller C.P., Roper T.J., 2004. Estimating population size by genotyping remotely plucked hair: the Eurasian badger. *J. Appl. Ecol.*, 41, 985-995.

Feigelson H.S., Rodriguez C., Robertson A.S., Jacobs E.J., Calle E.E., Reid Y.A., Thun M.J., 2001. Determinants of DNA yield and quality from buccal cell samples collected with mouthwash. *Cancer Epidemiol. Biomarkers Prev.*, 10, 1005-1008.

French D.J., Archard C.L., Andersen M.T., McDowell D.G., 2002. Ultra-rapid DNA analysis using HyBeacon probes and direct PCR amplification from saliva. *Mol. Cell. Probes*, 16, 319-326.

Goossens B., Waits L.P., Taberlet P., 1998. Plucked hair samples as a sources of DNA: reliability of dinucleotide microsatellite genotyping. *Mol. Ecol.*, 7, 1237-1241.

Healy P.J., Dennis J.A., Moule J.F., 1995. Use of hair root as a source of DNA for the detection of heterozygotes for recessive defects in cattle. *Aust. Vet. J.*, 72, 392.

Higuchi R., Von Beroldingen C.H., Sensabaugh G.F., Erlich H.A., 1988. DNA typing from single hairs. Nature, 332, 543-546.

Liu Y.S., Thomas R.J.S., Phillips W.A., 1995. Single-step direct PCR amplification from solid tissues. *Nucleic Acids Res.*, 23, 1640.

Meldgaard M., Bollen P.J.A., Finsen B., 2004. Non-invasive method for sampling and extraction of mouse DNA for PCR. *Lab. Anim.*, 38, 413-417.

Melton T., Dimick G., Higgins B., Lindstrom L., Nelson K., 2005. Forensic mitochondrial DNA analysis of 691 casework hairs. *J. Forensic Sci.*, 50, 73-80.

Menotti-Raymond M.A., David V.A., O'Brien S.J., 1997. Pet cat hair implicates murder suspect. *Nature*, 386, 774.

Mercier B., Gaucher C., Feugeas O., Mazurier C., 1990. Direct PCR from whole blood, without DNA extraction. *Nucleic Acids Res.*, 18, 5908.

Oberbauer A.M., Grossman D.I., Eggleston M.L., Irion D.N., Schaffer A.L., Pedersen N.C., Belanger J.M., 2003. Alternatives to blood as a source of DNA for large-scale scanning studies of canine genome linkages. *Vet. Res. Commun.*, 27, 27-38.

Panaccio M., Georgesz M., Hollywell C., Lew A., 1993a. Direct PCR from solid tissues without DNA extraction. *Nucleic Acids Res.*, 21, 4656.

Panaccio M., Georgesz M., Lew A.M., 1993b. FoLT PCR: a simple PCR protocol for amplifying DNA directly from whole blood. *Biotechniques*, 14, 238-243.

Pfeiffer I., Völkel I., Täubert H., Brenig B., 2004. Forensic DNA-typing of dog hair: DNA-extraction and PCR amplification. *Forensic Sci. Int.*, 141, 149-151.

Roon D.A., Waits L.P., Kendall K.C., 2003. A quantitative evaluation of two methods for preserving hair samples. *Mol. Ecol. Notes*, 3, 163-166.

Russo V., Dall'Olio S., Davoli R., Costelli M.B., Bigi D., 1996. Studio del *locus* Alotano nelle razze suine allevate in Italia. *Zootec. Nutr. Anim.*, 22, 33-38.

Sambrook J., Fritsch E.F., Maniatis T., 1989. *Molecular cloning: a laboratory manual*. Second edition. Cold Spring Harbor Laboratory Press, NY, USA.

Sandford A.J., Pare P.D., 1997. Direct PCR of small genomic DNA fragments from serum. *Biotechniques*, 23, 890-892.

Sloane M.A., Sunnucks P., Alpers D., Beheregaray L.B., Taylor A.C., 2000. Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censuring method. *Mol. Ecol.*, 9, 1233-1240.

Thomson D.M., Brown N.N., Clague A.E., 1992. Routine use of hair root or buccal swab specimens for PCR analysis: Advantages over using blood. *Clin. Chim. Acta*, 207, 169-174.

Walker A.H., Najarian D., White D.L., Jaffe J.M., Kanetsky P.A., Rebbeck T.R., 1999. Collection of genomic DNA by buccal swabs for polymerase chain reaction-based biomarker assays. *Environm. Health Perspectives*, 107, 517-520.

Wong L.-J.C., Lueth M., Li X.-N., Lau C.C., Vogel H., 2003. Detection of mitochondrial DNA mutations in the tumor and cerebrospinal fluid of medulloblastoma patients. *Cancer Res.*, 63, 3866-3871.