DNA from tissues of young mice is optimal for genotyping

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ABSTRACT

Background: Genotyping of mice is a common procedure in animal facilities. The aim of this study was to compare the quantity and quality of DNA extracted from samples obtained from young mice (YM; 10 d old) and adult mice (AM; 12 weeks old). We collected samples from the tail and ear of YM and AM. We also sampled blood, check cells (via buccal swabs), hair and fecal pellets of AM, and biopsied distal phalanx of YM. We isolated DNA using commercial kits and determined concentrations and purity by spectrophotometry. The integrity of DNA was evaluated by agarose-gel electrophoresis and polymerase chain reaction (PCR).
Results: DNA in all samples was amplified successfully but the intensities of bands after electrophoresis was heterogeneous. In general, tissues from YM yielded more DNA than those from AM, with differences being statistically significant for ear samples (38 ± 12 ng/μL for YM; 7 ± 3 ng/μL for AM; $P = 0.006$). In YM, the most DNA was obtained from ear and tail samples, with differences from the amounts obtained from phalanx samples being statistically significant ($P = 0.02$ and $P = 0.005$, respectively). In AM, the most DNA was obtained from tail and blood samples. Samples obtained by non-invasive sampling methods in adults resulted in a deficient DNA extraction.
Conclusions: The results of the present study do not support the previous recommendations for using non-invasive methods to genotype adult animals. The use of newborn tissue samples showed the highest efficiency for DNA extraction.

1. Introduction

Genetically modified (GM) mice are frequently used in biomedical research, and it is necessary to identify and/or confirm the genotype of animals, to eliminate genetic contamination and to control the quality of the animal colony. Such genotyping is a common procedure in many animal facilities.

Genotyping protocols should be fast and reliable and, currently, methods based on the polymerase chain reaction (PCR) and Southern blotting, are used most commonly [1]. Southern blotting is useful for the characterization of founders of a new transgenic line, and PCR is the most widely used method for routine genotyping during maintenance of colonies of genetically modified mice [2].

Successful amplification by PCR requires high-quality genomic DNA [3,4], and various biological tissues have been used as sources of DNA, for example, ear [5], tail [5,6], or phalanx biopsies [7,8]. Moreover, reliable labeling methods (for example, ear punching or phalanx removal) provide pieces of tissue that can be used for the isolation and genotyping of DNA, reducing animal procedures and the number of manipulations [2,9]. The quality of the DNA obtained from such tissues is usually good, but sampling procedures may cause pain and/or distress to the animals. In fact, some procedures, such as phalanx biopsy, are recommended only in neonatal [2,8] or pre-weaned mice [10].

Several methods of non-invasive genotyping have been proposed, such as sampling of saliva (by oral swabs) [11,12], hair [13] and fecal pellets [14,15], and methods for extraction of DNA from such animal samples are constantly improving [16].

Refinement of procedures for sampling and optimizing biological resources should enhance animal welfare and reduce costs.

The aim of this study was to compare the outcomes of DNA extraction from samples obtained from young and adult mice. Particularly, we valued the suitability of some non-invasive methods previously recommended.

2. Materials and methods

2.1. Animals

Swiss mice (HsdWin:NMRI) of both genders were purchased from a commercial breeder (Harlan Laboratories S.A., Barcelona, Spain) and were maintained at the authors' animal facilities during the entire study.

Mice were grouped as follows: 10 adult mice (12 weeks old; n = 5 for biopsies of tail, ear, and phalanx; buccal swabs, and collection of hair and fecal pellets; and n = 5 for blood samples), and 15 young

mice (10 d old; n = 5 for tail biopsies; n = 5 for ear biopsies; and n = 5 for phalanx biopsies).

Adult mice were housed in groups of five animals and young mice were housed with their mothers, in a controlled environment. The temperature in the room ranged from 20°C to 24°C, and the light/dark cycle was 12/12 h. All animals had unlimited access to water and standard rodent chow (2014 Teklad Global 14% Protein Rodent Maintenance Diet for adult mice and 2018 Teklad Global 18% Protein Rodent Diet for breeders; Harlan).

The study was performed in accordance with European Directive 2010/63/EU for the use of laboratory animals and with Spanish law (Real Decreto 1201/2005). As recommended by the Federation of European Laboratory Animal Science Associations (FELASA), mice in the animal facility were tested periodically to ensure that the colony remains free of pathogens such as *Mycoplasma pulmonis*, *Salmonella* sp., Sendai virus, Hantaan virus and Toolan Hl virus.

2.2. Sampling

All samples were taken from adult mice at the same time. Mice were restrained by a caretaker, who held them firmly by the back of the neck. Then, the researcher collected samples sequentially, as follows:

For each ear biopsy, a 2-mm-diameter diste was removed from the ear lobe with an ear punch.

For each tail biopsy, a piece less than 3 mm in length was cut from the tail tip with sharp surgical scissors and then a slight pressure was applied at the site of the cut for hemostasis.

To collect cells from buccal swab, a small cotton swab was introduced into the mouth of each animal and used to scrape the inner cheeks.

To collect hair samples we plucked hairs from the ventral body with sterile forceps.

For collection of fecal pellets, each animal was transferred to a clean cage and feces appeared within a few min. 100 to 110 mg of feces were collected in each case.

Blood samples were obtained from a separate group of adult mice by submandibular bleeding, according to a previously described technique [17]. The volume of each sample varied between 150 and 200 μL.

Animals were returned to their home cage immediately after completion of sampling.

Young mice were picked up and held by the nape of the neck, to mimic the way mothers carry their pups. Only one biopsy (from tail, ear or phalanx) was performed for each animal, using the same procedures described as those for adult mice. For each phalanx biopsy, no more than 2 mm of tissue was cut from the first phalanx with microsurgical scissors. After collection of tissue, animals were immediately returned to their home cage.

2.3. DNA extraction

All samples were stored at -20°C until analysis. DNA from samples of ear, tail and phalanx was isolated with a QIAamp DNA Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA was isolated from hair and buccal swab with the QIAamp DNA Mini Kit (Qiagen). The QIAamp DNA Stool Kit (Qiagen) was used for isolation of DNA from feces, and the Ultraclean DNA BloodSpin Kit (MoBio Laboratories, Carlsbad, CA, USA) was used to isolate DNA from blood. All procedures were performed according to protocols provided by the manufacturers, and, in all cases, the duration of each procedure was recorded.

2.4. Evaluation of the quantity and quality of isolated DNA

The amounts of DNA isolated from the various samples were determined by spectrophotometry with the NanoDrop ND-1000 system (NanoDrop Technologies, Inc., Wilmington, DE, USA). The purity of DNA was also determined spectrophotometrically from the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). A ratio of approximately 1.8 was accepted as evidence of the purity of DNA.

The integrity of isolated DNA was assessed by electrophoresis in $0.5\times$ Tris-Borate-EDTA buffer (TBE: Gibco-Invitrogen, Grand Island, NY, USA) in an agarose gel (1%) (Pronadisa, Madrid, Spain), that contained 3 μg/mL ethidium bromide. For these measurements we used a scale assigned to the various intensities of bands, as follows: ++ = strong; + = moderate; +↓ = low; +↓↓ = very low.

Amplification of DNA was evaluated by PCR directed towards amplification of a mouse housekeeping gene. We focused on a 140-bp sequence of the *k-ras* gene, using the forward primer 5′CCTGTGGTGG TTGGAAGCTGG-3′ and the reverse primer 5′CTGCCGTCCTTTACAAGC GCA-3′. The reaction mixture for PCR, with a total volume of 50 μL, contained a maximum of 200 ng of template DNA in a volume of 5 μL, plus 0.8 μM each primer, 0.2 μM deoxynucleoside triphosphate solution (Roche Diagnosis GmbH, Mannheim, Germany), 1.5 mM $MgCl_2$ (Roche Diagnosis GmbH), 5 μL of $10\times$ buffer, and 0.4 U of Taq DNA polymerase (Roche Diagnosis GmbH). Initial denaturation at 94°C for 10 min was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 30 s, and extension at 72°C for 45 s, with final extension at 72°C for 10 min. Temperature cycling was achieved with a DNA thermal cycler (iCycler; Bio-Rad, Hercules, CA, USA). The products of amplification were subjected to electrophoresis on a 2.5% agarose gel (Pronadisa) that contained 3 μg/mL ethidium bromide, in TBE buffer (Gibco).

2.5. Statistical analysis

Means of amounts of DNA and ratios were compared by Student's *t*-test for normal distributions and by the Kruskal–Wallis test for non-normal distributions. *P*-values of less than 0.05 were considered evidence of statistical significance.

Statistical analyses were performed with OpenEpi (Open Source Epidemiologic Statistics for Public Health, Version 2.3.1; www.OpenEpi.com).

3. Results

3.1. Efficacy of DNA-extraction procedures

The extraction of DNA from blood samples took 40 min. By contrast, the isolation of DNA from the tail, ear, phalanx and hair samples took 16 h, including an overnight incubation.

The final elution volume was 200 μL in the case of DNA extracted from the tail, ear, phalanx and blood samples; 150 μL in the case of DNA extracted from fecal pellets; and 50 μL in the case of DNA from hair and buccal swabs.
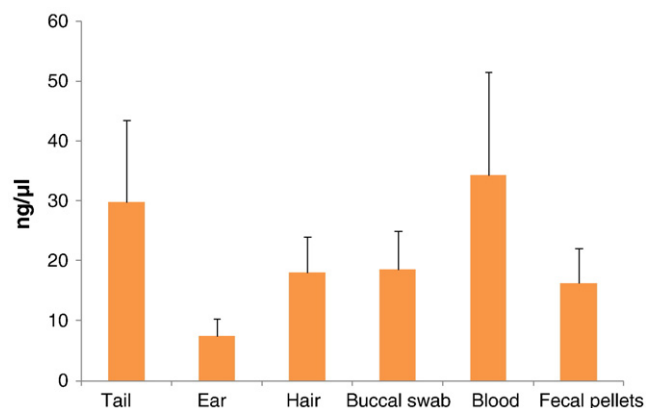

Fig. 1. Histogram showing the mean concentrations of DNA obtained from samples from adult mice, as indicated. Values are means ± S.D. of results from 10 adult mice.
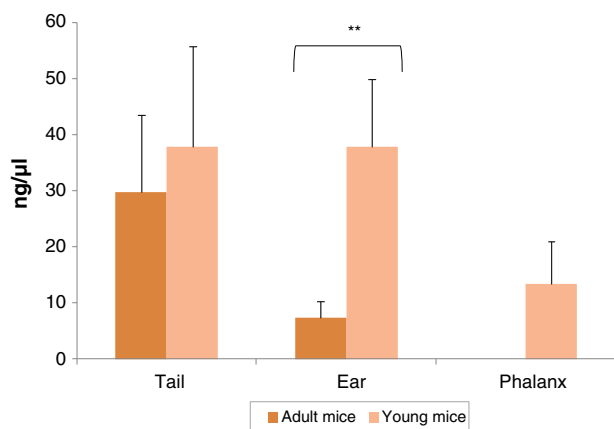
Fig. 2. Histogram showing mean concentrations of DNA obtained from indicated samples from young and adult mice. Values are means ± S.D. of results from 10 adult mice and 15 young mice. **$P = 0.006$.

Detectable amounts of DNA were obtained from all samples (Fig. 1 and Fig. 2). In adult mice, the largest amounts were obtained from blood (34 ± 17 ng/μL; mean ± S.D. n = 5) and tail samples (30 ± 14 ng/μL; n = 5). The lowest amounts were obtained from ear samples (7 ± 3 ng/μL; n = 5). Results for blood and tail samples were significantly different to those obtained for ear samples ($P = 0.03$ and $P = 0.02$, respectively).

In general, samples from young mice yielded more DNA than those from adults, and the difference was statistically significant for ear samples ($P = 0.006$; Fig. 2).

In young mice, the most DNA was obtained from tail (38 ± 18 ng/μL, n = 5) and ear samples (38 ± 12 ng/μL, n = 5), and the differences with respect to phalanx samples (13 ± 8 ng/μL, n = 5) were significant ($P = 0.02$ and $P = 0.005$, respectively).

Pure DNA ($A_{260}/A_{280} \approx 1.8$) was obtained from samples of the tail, ear, blood and feces from adult mice, and from all samples (tail, ear and phalanx) from young mice. The purity of the DNA obtained from hair and from buccal swabs of adult mice was the lowest (0.80 ± 0.02 and 0.83 ± 0.02; n = 5 and n = 5, respectively). Young mice yielded larger ratios than adult mice, with statistically significant difference for samples of tail ($P < 0.0001$).

The integrity of DNA from adult mice, as evaluated by electrophoresis, was heterogeneous (Table 1). The most intense bands were observed in the case of DNA from tail and blood. The DNA from hair, buccal swabs and feces yielded weak bands. All samples of DNA from young mice yielded intense bands.

We were able to amplify DNA from all samples by PCR, but the intensity of the electrophoretic bands of products was variable, in particular in the case of DNA from adult mice (Table 1). The most intense bands were observed after amplification of DNA extracted from samples tail, ear and blood. Products of PCR with DNA from hair, buccal swabs and fecal pellets were visible as only weak bands (Fig. 3). However, all samples of DNA from young mice yielded intense bands.

4. Discussion

There is a general interest in the pain and/or distress provoked by sampling tissues for genotyping of GM because most methods are invasive. However, non-invasive methods have also been described recently [11,12,13,14,15]. Moreover, inadequate amounts and/or poor quality of DNA extracted for genotyping can be a problem if large amounts are required and if animals have to be subjected to repeat analyses.

Several groups have recommended the use of pre-weaned mice for genotyping, in particular if biopsies are taken from the tail or phalanx [2]. Our results support this recommendation since; in general, biopsies from young mice resulted in larger amounts of pure DNA than those from adult mice.

Tail biopsy appears to be the most common sampling method for genotyping, both in young and adult mice [2]. In a recent study, it has been suggested that in a neonatal mouse or rat, particularly prior to approximately 12 d of age, a nociceptive stimulus may not result in the conscious perception of pain due to the lack of a competent pain pathway at this age [18]. At 14–17 d after birth, mice have less ossified tails and samples can be smaller than those from adult mice [6]. We took 2-mm tail biopsies from both young (ten d old) and adult mice, and the quality and quantity of DNA obtained were excellent in all cases. Indeed, commercial kits allow efficient isolation of good-quality DNA from small and varied samples. The major disadvantage of the tail snip is that it does not allow simultaneous labeling of animals and it remains necessary to label them by some other method.

Ear biopsy is routinely used in many laboratories for tagging and genotyping adult mice. A disk of approximately 2 mm in diameter from the pinna ear is sufficient for isolation of DNA and amplification by PCR [2,9]. The greatest advantage of this procedure is that it allows the simultaneous tagging of the mouse and the removal of a sample for genotyping. However, the use of an ear biopsy as a source of DNA in mice is relatively rare with two invasive procedures being performed instead of one [2]. Although ear sampling is an invasive procedure, the risk of hemorrhage and pain is minimal because the pinna ear contains mainly cartilage, and biopsy can be performed without anesthesia. We did note, however, that young mice showed a slight shake when ear samples were taken. All ear samples from young and adult mice yielded amplifiable DNA. However, in young mice, the ear is not yet erect and the pinna is still too small to allow different types of labeling. Thus, ear biopsy appears to be not suitable for labeling large numbers of mice younger than 15 d [2,9].

Phalanx biopsy has been used to label, pre-weaned mice and also as a source of samples for genotyping [8]. In young mice the ossification process is not yet complete and phalanx biopsy can be performed without pain and distress [7,8]. Recent FELASA guidelines [2] recommend that, for phalanx biopsy, mice should be not more than approximately 7 d old. However, by developmental and behavioral assessments, other authors have recently shown that toe clipping of mice as old as postnatal d 17 is an acceptable method [10]. Moreover, they have suggested that when both permanent identification and Southern blot analysis are needed, toe clipping and toe biopsy should be performed simultaneously between postnatal d 10 and 17 [10]. In the present study, we used 10-d-old mice, removing 2 mm long samples of phalanx, and no animals showed movements that

Table 1
Relative intensities of bands of DNA obtained by electrophoresis after extraction of DNA (1% agarose gel) and after PCR (2.5% agarose gel). ++ = strong; + = moderate; +↓ = low; +↓↓ = very low.

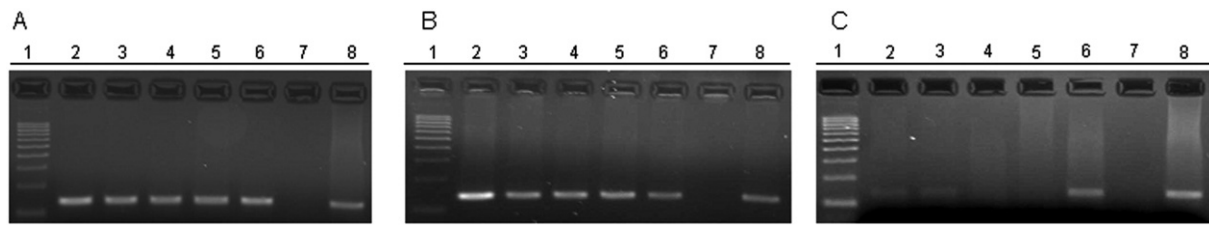|	Adult mice						Young mice		
	Tail	Ear	Blood	Buccal swab	Hair	Feces	Tail	Ear	Phalange
Electrophoresis after DNA extraction	++	+	++	+↓↓	+↓↓	+↓	++	++	++
Electrophoresis after PCR	++	++	++	+↓↓	+↓↓	+↓	++	++	++

Fig. 3. Photographs of three agarose gels showing the results of amplification by PCR of a 140 bp *k-ras* sequence in DNA obtained from samples from adult mice: A) ear samples; B) blood samples; C) fecal pellets. Lanes 1: 100-bp DNA ladder; lanes 2 to 6: samples from individual animals; lanes 7: negative controls (water); lanes 8: positive controls (DNA obtained from NIH-3T3 mouse cell line).

suggested pain during the procedure. In addition, we were able to efficiently amplify the DNA by PCR in all cases and, thus, these results support the suggestion of using this sampling technique as the method of choice to genotype and in parallel to label newborns [2].

Extraction of DNA from blood samples from adult mice is rapid and efficient and provides high quality DNA. If the volume of blood collected is kept to a minimum (no more than 10% of the total blood volume), this technique has the advantage of being repeatable. However, proper training is required to avoid inaccurate puncture and/or hemorrhages. In the present study, no animal showed hemorrhaged or even bled moderately. However, the procedure appeared to be the most painful for mice and the most uncomfortable for the researcher. Moreover, blood sampling does not allow labeling of mice for identification and, thus, a second method for identification is required.

While buccal swabbing has been described as non-invasive method [2], such sampling appeared to be as stressful as sampling from the tail or ear. Moreover, we obtained only relatively small amounts of DNA in terms of both volume and concentration. There are, however, reports [11,12] of the reliable and efficient isolation of DNA from buccal swabs by other methods that yield free from inhibitors of PCR.

Hair samples, yielded results to similar those obtained with buccal swabs. Hair sampling is a fast and non-invasive method for genotyping mice but, unfortunately, there is a high risk of cross-contamination between samples from different animals, because hairs stick electrostatically to instruments [5]. Thus, hair is only recommended for genotyping small numbers of animals with an efficient method for amplification by PCR.

Genotyping of DNA from fecal pellets would appear to have advantages, for example, it is simple, non-invasive and painless, also allowing repeat sampling whenever necessary [14,15]. However, sampling might not be entirely stress-free, because the time for collection of each sample can range from 20 to 30 s. In addition, fecal pellets contain many inhibitors of PCR, such as bilirubin, bile salts and calcium ions [19]. While commercial kits usually remove such inhibitors, DNA from intestinal microorganism in the feces might contaminate the mouse's genomic DNA. Our results suggested the presence of stool microorganisms in samples, since PCR yielded weak bands of the desired products in spite of the fact that the quality and quantity of DNA obtained from feces were acceptable.

In summary, tissues from young mice tended to yield DNA in higher quantities and of higher quality than that from adult mice. In young mice, biopsies for genotyping can be performed simultaneously with labeling for identification via the ear or phalanx. In adult mice, we obtained the best results from blood and tail samples, although the major disadvantage of the two techniques is that they do not allow simultaneous labeling of animals. For a variety of reasons, DNA obtained by non-invasive sampling methods, such as fecal pellets, hair and buccal swabs, yielded the weakest bands of products of amplification, by PCR, of a mouse housekeeping gene.

In conclusion, the results of the present study do not support the use of non-invasive methods for sampling to genotype. The use of newborn tissue samples showed the highest efficiency for DNA extraction with, possibly, the minimum pain and stress for animals.

5. Competing interests

Non-financial competing interests

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