Implications for the use of horse hair roots as a DNA source for microsatellite typing

T. Ząbek, A. Radko, E. Słota

Immuno- and Cytogenetics Department, National Research Institute of Animal Production, Balice, Poland

ABSTRACT: Hair roots are a very attractive source of DNA for microsatellite-based parentage control of breeding animals. However, unlike blood samples, irregular DNA typing results have been observed in assays utilizing hair follicles. The amount of starting material and DNA preparation method are the crucial factors. In order to improve DNA typing results for horse hair roots, two quick preparation methods and additional purification steps were evaluated. PCR efficiency for each approach was expressed as percentage of samples with complete DNA profiles for 12 horse microsatellites. The lowest percentage (22%) of complete DNA profiles was obtained for samples prepared by the proteinase K digestion method. The best genotyping results (94%) were achieved after phenol-chloroform extraction of DNA from samples prepared by the proteinase K digestion method. Direct cleanup of DNA samples with an ethanol-sodium acetate mixture gave comparably good results of microsatellite genotyping (91%). DNA preparation from hair roots with proteinase K digestion followed by DNA purification with ethanol was chosen as the most efficient approach for horse DNA typing under parentage testing.

Keywords: hair follicles; short tandem repeats; multiplex PCR

Progress in haemogenetic techniques applied for individual identification and parentage verification enabled investigations to be performed at the DNA level. For around 15 years, PCR amplification of microsatellite sequences has been a fundamental technique used for these purposes. A broad availability of genetic material from different biological sources and the introduction of automated DNA fragment analysis made the above approach very attractive.

Plucked hair roots are an excellent DNA source for a great number of animals involved in routine parentage testing. Two major populations of hair roots can be readily identified in any individual: one in the 'resting phase' (telogen), the other in the 'growing phase' (anagen). Hair roots in anagen, preferably with sheaths attached, are required for assays. Hair roots in telogen lack nucleated cells and cannot serve as a useful source of genomic DNA (Healy et al., 1995). The collection, transportation and storage of hair bulbs do not require any special conditions and any financial costs in contrast to other tissues such as blood. Despite the attractiveness of hair bulbs as a DNA source, the quality of microsatellite profiles for DNA samples from roots is usually lower than that typical of DNA isolated from blood specimens (Ellegren et al., 1992). Thus in assays with the use of DNA prepared from hair roots, genotyping errors may occur for weaker amplified microsatellites in a multiplex reaction.

In this paper we present several methods of DNA preparation from hair roots that have been tested to choose an optimum one for reducing PCR related problems in reactions of multiplex type.

MATERIAL AND METHODS

Genomic DNA was prepared from hair roots of about 200 half-bred Anglo-Arabian horses regardless of sex and age. Around 20 hairs were plucked

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from the mane and stored in a dark place at room temperature. The collected material was divided into 5 groups from which DNA was isolated using different methods:

1. DNA preparation through overnight proteinase K digestion during incubation (56°C in water bath) of 2 hair roots (30 samples) in a lysis buffer-K (10mM Tris-HCl pH 8.3, 50mM KCl, 0.5% Tween 20), as modified according to the method of Kawasaki (1990). Proteinase K was finally inactivated through 10 min. incubation of DNA samples at 95°C.

2. DNA isolation from 1–4 hair roots (30 samples) using the first preparation method.

3. Modified DNA preparation procedure according to Drissing et al. (1996) when 4 hair roots (40 samples) were incubated at room temperature for 5 min in 2.5 µl of 0.1M NaOH, after which 22.5 µl of 0.02M Tris-HCl, pH 7.4 was added.

4. DNA extraction from DNA samples prepared using method No. 1 (50 samples) with phenol: chloroform:isoamylalcohol (25:24:1) mixture and recovery of nucleic acids by precipitation with absolute ethanol in the presence of 0.3M sodium acetate (pH 5.2). DNA was finally washed with 70% ethanol (Sambrook et al., 1989) and suspended in 25 µl of Tris-EDTA, pH 8.0.

5. Recovery of nucleic acids directly from DNA samples prepared by method No. 1 (50 samples) by precipitation with ethanol-sodium acetate and final washing with 70% ethanol (Sambrook et al., 1989).

Photometric determination of nucleic acids in the samples was performed with a BioPhotometer (Eppendorf AG, Hamburg, Germany). The absorbance at 260 nm (A_{260}) for nucleic acids and at 280 nm (A_{280}) for proteins was measured. The ratio between reading values ($A_{260/280}$) determined the purity of DNA samples when the range of 1.8 to 2.0 indicated the required purity level for double stranded DNA (dsDNA) (Sambrook et al., 1989). Measured values were used to determine approximate DNA yield from hair root samples by the use of different preparation methods.

Analysis of microsatellite polymorphism:

Genomic DNA was the template (1 µl) for two multiplex PCR reactions (14 µl each) of 12 microsatellite markers (8-plex: *AHT4, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, VHL20* and 4-plex: *AHT5, ASB2, HMS2, HTG10*) (StockMarks® for Horse and Cattle Paternity PCR Typing Kits, 2002). Amplified PCR products were separated on 4% polyacrylamide gel during electrophoresis on an ABI 377 sequencer (Applied Biosystems, Foster City, California, USA). Genetic profiles of the studied individuals were analysed in Genotyper 2.0 software and used for evaluation of PCR efficiency depending on the DNA isolation method. The intensity of fluorescent signal (relative fluorescent units) correlated with the high peaks in Genotyper plots was the measure of PCR quality of microsatellite markers. Percentage of samples with complete DNA profiles in the 12 microsatellite loci expressed the efficiency of PCR multiplex reaction with the use of the DNA preparation method from roots.

RESULTS AND DISCUSSION

The common problem for the DNA template from hair roots is missing profiles for some mic-

Table 1. PCR efficiency, amount and purity of DNA samples from hair roots for different methods of DNA preparation

DNA preparation method	Samples with complete DNA profile (%)	dsDNA concentration (ng/µl)		A260/280	
		mean ± S.E.	S.D.	mean ± S.E.	S.D.
Proteinase K digestion (1–4 roots)	22	188.0 ± 9.1	53.0	0.78 ± 0.01	0.06
Proteinase K digestion (2 roots)	48	218.5 ± 7.6	35.8	0.77 ± 0.01	0.05
Drissing method	65	61.5 ± 5.1	55.4	1.16 ± 0.03	0.34
Phenol:chloroform extraction	94	37.6 ± 2.6	13.7	1.89 ± 0.02	0.10
Ethanol precipitation	91	29.5 ± 1.4	9.26	1.87 ± 0.05	0.23

S.E. – standard error; S.D. – standard deviation

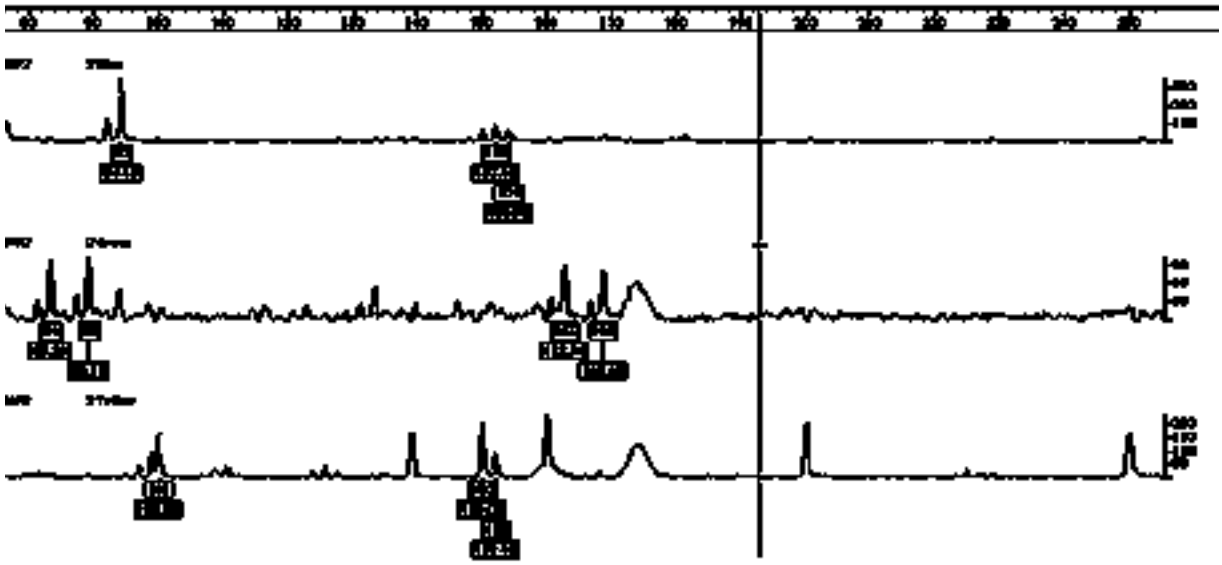

Figure 1. DNA profile of a sample prepared using the proteinase K digestion method

Bp – base pairs (horizontally); RFU – relative fluorescent units of PCR products (vertically)

rosatellite loci or a complete lack of all PCR products (Figure 1). The variable DNA concentration in DNA extracts (large standard deviation for dsDNA concentration in Table 1) seems to be the main reason for PCR related problems depending on the number and type of selected hair roots. Therefore our aim was to establish approximate DNA amounts to obtain good PCR results. Substantially lower percentages of complete DNA profiles for hair root samples were obtained with the use of the common proteinase K digestion method and a rapid method described by Drissing et al. (1996) (Table 1). The main problem with the use of the proteinase K method was the high content of impurities ($A_{260/280}$ < 1) and the excess of DNA in the samples (~200 ng/µl in relation to 25 ng/µl for

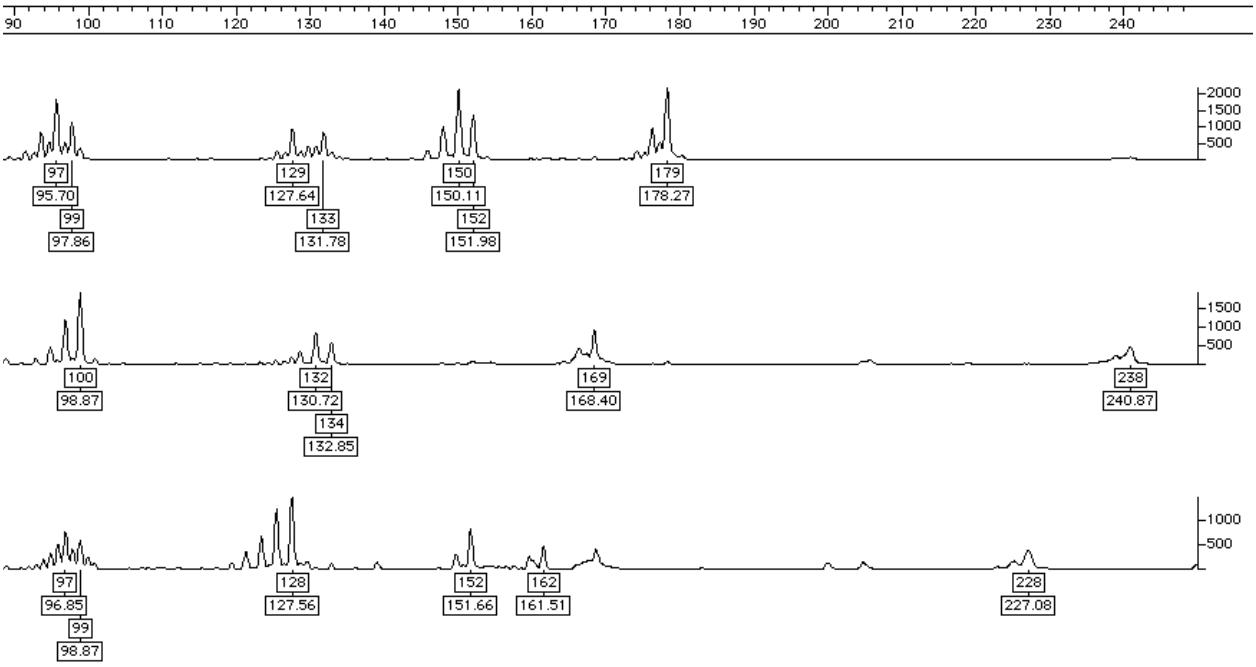

Figure 2. DNA profile of a sample purified with a phenol-chloroform mixture followed by ethanol precipitation

Bp – base pairs (horizontally); RFU – relative fluorescent units of PCR products (vertically)

the control DNA sample), which inhibited the amplification of microsatellite loci with alleles larger in size (Figure 1). In the Drissing method, DNA was often released in smaller quantities from cells together with active endonucleases which caused rapid DNA degradation. Thus longer storage of such samples was impossible, which limited their use in disputed parentage cases.

As expected, the best quality of DNA profiles and the highest percentage of complete genotype data were typical of DNA extracted using the phenol: chloroform mixture followed by the recovery of DNA with sodium acetate-ethanol precipitation (Figure 2). This procedure is widely used in forensic (Kochl et al., 2005) and archaeological studies (Kalmar et al., 2000) as a powerful method allowing recovery of DNA from degraded biological samples. High purity of DNA extracts and DNA concentration close to that required in the multiplex reaction (Table 1) maximised the yield of amplified products in all microsatellite loci. Although this method is most efficient for different molecular applications, it is time consuming and difficult to apply in numerous DNA samples under routine parentage control. To facilitate the procedure of DNA purification step, we used only the recovery of DNA with sodium acetate-ethanol precipitation. In DNA samples purified with ethanol in the presence of inorganic salt, the quality of DNA and percentages of complete DNA profiles were close to DNA extracted with the phenol:chloroform mixture (Table 1). The use of the ethanol precipitation step was enough to remove inhibitors strongly affecting the quality of PCR products of the multiplex type.

The use of two hair roots in anagen as the starting material and the preparation of DNA extracts with the proteinase K digestion method, followed by DNA precipitation with ethanol, were finally found as the most efficient approach providing DNA profiles of good quality (Figure 2). Despite the small number of cases with wrong selection of hair roots, all tested individuals were successfully genotyped in the 12 microsatellite loci. In contrast to blood as a DNA source for genotyping numerous samples, DNA derived from hair roots requires an additional purification step to minimize repeated testing and additional costs resulting from a great number of reactions.

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Corresponding Author

Dr. Tomasz Ząbek, National Research Institute of Animal Production, Immuno- and Cytogenetics Department, Krakowska 1, 32-083 Balice, Poland
Tel. +48 122 588 399, e-mail: tzabek@izoo.krakow.pl