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COMPARATIVE ANALYSIS OF TOTAL DNA ISOLATION PROCEDURE FROM BLOOD AND HAIR FOLLICLE SAMPLES IN GOATS

ANALIZA COMPARATIVĂ A PROCEDURII DE IZOLARE DE ADN TOTAL DIN PROBE DE SÂNGE ȘI FOLICULI PILOȘI DE CAPRE

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ABSTRACT | REZUMAT

The isolation of the DNA from biological samples is one of the most important steps in molecular biology methods. Although it is a nonspecific step, being a total DNA isolation, the amount of genetic material is essential for the specific amplification of the targeted DNA fragment. At this moment there are numerous commercial DNA isolation kits. Most of these kits isolate the DNA from a multitude of biological samples: whole blood, tissues, biological fluids, cell cultures, swabs, etc. However, in optimizing a molecular biology method, the type of biological sample will also be taken into account, as the quantity and quality of DNA may differ depending on the type of sample used in the extraction. The present study aimed at the comparative analysis of a method of isolating total DNA from whole blood and hair follicles from goats, in order to optimize a non-invasive method of sampling biological material. We used a commercial kit to isolate DNA for both whole blood and hair follicle samples. In the end, a fluorescence-based method for the quantitative determination of DNA in probes was performed. The results show that the genetic material obtained from hair follicle samples was between 10 ng / μL and 50 ng / μL, while the amount of DNA obtained from whole blood samples was between 19.3 ng / μL and 57 ng / μL. Given the DNA amounts obtained from the two types of biological samples, whole blood and follicles hair, we can appreciate that in both types of biological samples the DNA concentrations are enough for the specific amplification of 1200bp DNA fragments. In conclusion, the hair follicles of goat can be used as a non-invasive method of sampling biological material for the genomic DNA isolation.

Keywords: total DNA extraction, DNA quantity, hair follicles, blood samples, goat

Izolarea ADN-ului din probele biologice este una din cei mai importante etape ale metodelor de biologie moleculară. Deși este o etapă nespecifică, fiind o izolare totală de ADN, cantitatea de material genetic este esențială pentru amplificarea specifică a fragmentului țintă de ADN. Pentru realizarea acestei etape, au fost realizate numeroase kituri comerciale de izolare ADN. Majoritatea kiturilor pot izola ADN din mai multe tipuri de probe biologice: sânge integral, țesuturi, fluide biologice, culturi celulare, tampoane etc. Totuși, în optimizarea unei metode de biologie moleculară se va avea în vedere și tipul probei biologice, deoarece cantitatea și calitatea ADN-ului pot diferi în funcție de tipul probei folosite la extracție. În studiul de față s-au analizat comparativ metodele de izolate a ADN-ului total din sânge și foliculi piloși de capră, în vederea optimizării unei metode non-invazive de obținere a ADN-ului genomic necesar genotipării lor. Pentru obținerea ADN-ului din probele de sânge și foliculi piloși, s-a utilizat un kit de extracție disponibil comercial. În final, a fost efectuată o metodă bazată pe fluorescență pentru determinarea cantitativă a ADN-ului din probe. Materialul genetic obținut din probele de foliculi piloși testate a variat între 10 ng/μL și 50 ng/μL, în timp ce în probele de sânge au variat între 19,3 ng/μL și 57 ng/μL. Având în vedere cantitățile de ADN obținute în cele două tipuri de probe biologice, sânge și foliculi piloși de capră, putem aprecia că în ambele tipuri de probe biologice concentrațiile de ADN sunt suficiente pentru amplificarea specifică a unor fragmente ADN de 1200 perechi de baze. In concluzie, foliculii piloși de capră pot fi utilizați ca metodă neinvazivă de prelevare a materialului biologic pentru izolarea ADN-ului genomic.

Cuvinte cheie: extracție ADN total, cantitatea ADN, foliculi piloși, probe de sânge, capră

The isolation of the DNA from biological samples is extremely important for molecular biology methods. Although it is a nonspecific step, being a total DNA isolation, the amount of genetic material is essential for the specific amplification of the DNA target fragment. To accomplish this step, several DNA isolation kits were achieved and commercialized. Most of these kits isolates the DNA from various biological samples: blood, tissues, biological fluids, cell cultures, swabs, etc. The appropriate kit will be used considering the

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fact that the amount and quality of DNA may also differ depending on the biological sample that was used (2, 7). The first step of the isolation of DNA is the cells lysis and then DNA solubilization. In order to remove the lipids, RNA, and proteins is mandatory the chemical or enzymatic step. DNA extraction method include: phenol–chloroform method, salting out, proteinase K treatment, and silica-gel membrane adsorption (2, 4, 7). In generally, there are four mandatory steps for the DNA isolation: cell lysis, cellular proteins dehydration and precipitation, DNA separation from the cellular proteins and other components, and precipitation of the DNA (3). A high and very good quality of the DNA can be isolated from whole blood samples (8). An alternative to the whole blood samples (an invasive DNA collection method), can be the animal hair samples (a less-invasive DNA collection method), collected with follicles where the quantity of the DNA is higher even if in the literature was described methods that can isolates DNA from the hair shafts (5, 6). DNA isolation from biological hair samples has been tested and used successfully in human medicine (9, 13, 15), but the method used in human hair samples can also be successfully applied in animal hair samples. Hair sample as a DNA source is a very valuable sample for non-invasive method either for human and animals' population. Hair can be used in genetic studies in order to identify the breed of an animal, paleontological research, the genotype for the susceptibility to diseases etc (6, 9). The present study aimed for the comparative analysis of a method of isolating total DNA from blood and hair follicles from goat, in order to optimize a non-invasive method of sampling biological material. The optimization of a DNA isolation method from goat hair samples is necessary for a future step of determining scrapie susceptible genotypes by the sequencing method.

MATERIALS AND METHODS

Sampling

DNA isolation was performed from whole blood samples collected on K3-EDTA and hair samples with follicles from 20 Carpathian breed goats. The goats were from the south-eastern part of Romania. Their ages were between 2 years and 15 years. From a total of 20 animals, 18 were females and 2 males. Blood samples were collected in 5 ml tubes containing K3-EDTA and stored at -20°C until DNA extraction. The hair samples were collected in order to have 30-40 hair follicle that provide the required amount of DNA for extraction. After sampling, the hair follicles were refrigerated at 1-3°C until DNA extraction. Both, blood and hair follicle samples from the same animals were identified in order to make a comparative quantification of the total amount of DNA extracted from each type of biological sample. After DNA isolation, the amount of total DNA extracted was quantified with a fluorometer.

Reagents and materials

DNA extraction was made with PureLink Genomic DNA Kits (10) by using different protocols for whole blood and hair follicles samples. Quantification of total DNA was made using Qubit dsDNA HS Assay Kits (11, 12) with the Qubit 4 Fluorometer. Others necessary materials were: 96–100% ethanol, sterile DNase–free microcentrifuge tubes 1.5 ml, heat block which provides a 56°C, Petri dishes, and disposable scalpels.

DNA extraction from blood samples using PureLink Genomic DNA Kits (Invitrogen)

Total DNA extraction from blood samples was performed according to the protocol described below (Table 1). After sampling, the blood was vortexed and 200l was transferred to a 1.5 ml sterile Eppendorf tube. The first step in DNA extraction is represented by cell lysis which will be achieved by adding proteinase K and PureLink Genomic Lysis / Binding Buffer. RNase A is required in DNA extraction to destroy any amount of RNA and thus obtain a purified DNA extract. RNase A is supplied with the kit and an RNase digestion step is made during sample lysis step. The Proteinase K is used for efficient lysis of blood cells which is mandatory to isolate the DNA. Then the Eppendorf tube is incubated at 55°C for 10 minutes to promote protein digestion. After the incubation step, ethanol 96º-100º will be added for the purpose of the cell lysis step. The next step is the binding DNA. The kit that was used have a PureLink® Spin Column with a silica membrane to isolate the DNA. The PureLink® Genomic DNA Kits is doing the DNA binding with a silica-based membrane in the presence of chaotropic salts. The lysate ~640l was transfer on column and centrifuge at 10,000 x g, 1 min. The washing step was made by 2 washing buffers provides by the kit. The final step was the DNA elution. In our case, the elution was done in 50 l PureLink Genomic Elution Buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA).

According to the manufacturer's specifications, by using 50 µL or 100 µL elution buffer volume, it will recover ~90% and 80% of genomic DNA, respectively. The difference of the DNA extraction protocol from the blood compared to that of other tissues is the use of a single reagent that has the role of both digestion and DNA binding, PureLink Genomic Lysis/Binding Buffer, as well as the incubation time for cell lysis which in the case of blood only 10 minutes and in the case of other tissues it is one hour. In our study, we performed DNA extraction from the whole blood according to the protocol given in table 1 in accordance with the manufacturer's recommendations.

Table 1

DNA Isolation from blood using PureLink Genomic DNA Kits (Invitrogen)

Reagents	µl/sampl
The blood sample collected on EDTA is added to an Eppendorf	200 µl
Proteinase K (mixing and vortexing)	20 µl
RNase A	20 µl
Vortex and incubate at room temperature for 2 minutes	
PureLink Genomic Lysis/Binding Buffer (mixing and vortexing)	200 µl
Incubate at 55 °C for 10 minutes with vortexing	
Ethanol 96°-100° (vortexing and mixing) 5 seconds	200 µl
Transfer the lysate ~ 640 ul on columns - centrifugation at	
Remove the supernatant and transfer the colony to a new tube	
Wash Buffer 1	500 µl
Centrifuge at 10,000xg, 1 min.	
Wash Buffer 2	500 µl
Centrifuge at maximum speed 3 min.	
Transfer the colony to a 1.5 ml tube	
PureLink Genomic Elution Buffer	50 µl
Incubate at room temperature, 1 min.	
Centrifuge at maximum speed, 1 min	
The column is removed and the DNA is stored at -20 °C	

DNA extraction from hair follicles samples using PureLink Genomic DNA Kits (Invitrogen)

Total DNA extraction from hair follicles samples was performed according to the protocol described below (Table 2). On a Petri dish, 30-40 hair follicles/ sample were cut with a sterile knife and put into a 1.5 ml Eppendorf tube. Pure Link Genomic Digestion Buffer and proteinase K were added and the tubes were incubated at 55 °C with vortexing for 1 hour. After the incubation there was performed a centrifugation for 3 minutes at maximum speed and then the supernatant was transferred to a new Eppendorf tube. Then it was added RNase A which is required in DNA extraction to destroy any amount of RNA and thus obtain a purified DNA extract. The next step is the binding DNA. The kit that was used have a PureLink® Spin Column with a silica membrane to isolate the DNA. The PureLink® Genomic DNA Kits is doing the DNA binding with a silica-based membrane in the presence of chaotropic salts. The lysate (~ 640l) was transfer on column, centrifuged at 10,000xg for 1 min, and after there was added PureLink Genomic Lysis/Binding Buffer and Ethanol 96°-100°. The washing step was made by 2 washing buffers. DNA elution was done in 50 l Pure Link Genomic Elution Buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA). The difference of the DNA extraction protocol from the blood compared to that of hair follicles sample is the use of a single reagent that has the role of both digestion and DNA binding (PureLink Genomic Lysis/Binding Buffer in the case of blood samples). In the hair follicles samples was used a digestion buffer before to be added the lysis/binding buffer.

The incubation time for the cell lysis in the blood sample case was 10 minutes and for the hair follicles was one hour.

Table 2

DNA Isolation from hair follicle using PureLink Genomic DNA Kits (Invitrogen)

Reagents	µl/sample
Hair follicles - cut 30-40 hair follicles, crush it with a sterile	
Pure Link Genomic Digestion Buffer	200
Proteinase K (mixing and vortexing)	20
Incubate at 55 °C with vortexing- 1 hour	
Centrifuge lysed for 3 minutes at maximum speed	
Transfer the supernatant to a new Eppendorf tube	
RNase A (mixing)	20
Vortex and incubate at room temperature for 2 minutes	
PureLink Genomic Lysis/Binding Buffer (vortexing and mixing)	200
Ethanol 96°-100° (vortexing and mixing) 5 seconds	200
Transfer the lysate ~ 640 ul on columns.	
Remove the supernatant and transfer the colony to a new tube	
Wash Buffer 1	500
Centrifugation at 10,000xg for 1 min.	
Wash Buffer 2	500
Centrifuge at maximum speed 3 min.	
Transfer the column to a 1.5 ml tube	
PureLink Genomic Elution Buffer	50
Incubate at room temperature 1 min	
Centrifuge at maximum speed 1 min	
The colony is removed and the DNA is stored at -20 °C	

DNA quantification

Quantification of total DNA was made using Qubit dsDNA HS Assay Kits (11, 12) with the Qubit 4 Fluorometer. The kit has optimal performance when all the solution that will be used are at the room temperature between 22 °C and 28 °C because the temperature fluctuations can influence the accuracy of the assay. The fluorometer method is measuring the intensity of the signal from fluorescent dyes which are bound to the DNA molecule. Qubit 4 Fluorometer is using curve-fitting algorithms in order to develop a calibration curve that is used than as a standard sample with a known concentration. There are 2 standards that it will be the first performed to the Qubit 4 Fluorometer. A sample with an unknown concentration of DNA it will be calculated by comparing the relative fluorescence units (RFUs) of the sample to the RFUs of the standards used in calibration. Contaminants that can be in the sample as: salts, RNA, solvents, detergents, and proteins are very good tolerated by this method. There are two types of Invitrogen Qubit dsDNA assay kits: Invitrogen Qubit dsDNA High-Sensitivity (HS) Assay Kit and Invitrogen Qubit dsDNA Broad-Range (BR) Assay Kit. The first one is for samples with a low concentration of dsDNA and the second is for broad range DNA concentration. In this study was used the dsDNA High-Sensitivity (HS) Assay Kit. The protocol for the quantification began with the preparing of the working solution by diluting the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS Buffer. The final volume for each sample must be 200 µL (Table 3). For the standard was used 190 µL Qubit working solution and for

Table 3

The protocol for quantification the Standards and the Samples

Working solution volume		Standard 1	10 µL	Sample 1	2 µL
		Standard 2	10 µL	Sample 2	2 µL
Qubit dsDNA HS buffer	199 µL	Working solution	190 µL	Working solution	198 µL
Qubit dsDNA HS reagent	1 µL				
Total	200 µL	Final volume for reading 200 µL			

the sample 198 µL Qubit working solution. Qubit reagent was used 10 µL for each standard and 2 µL for the samples with a final volume of 200 µL (Table 3). Then was mix by vortexing 2-3 seconds, incubated at room temperature for 2 minutes and proceed to reading to the Qubit 4 Fluorometer. First were read the standard and then the samples. The results were expressed in ng/µL.

RESULTS AND DISCUSSIONS

The results show that the genetic material obtained from hair follicle samples was between 10 ng/µL and 50 ng/µL, while the amount of DNA isolated from whole blood samples was between 19.3 ng/µL and 57 ng/µL. The DNA quantity from blood samples was higher than the DNA quantity from hair follicles in all 20 samples (Fig. 1).

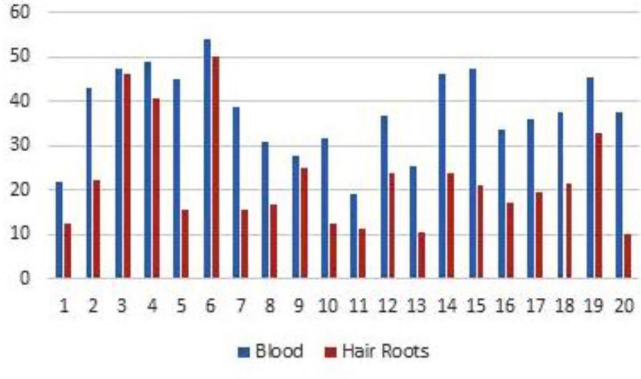

Fig. 1. The total DNA amount quantities in whole blood and hair follicle samples from 20 goats

The differences between the two types of samples in terms of the DNA amount are smaller in the samples 3, 4, 6, 9, and 11. In blood sample 3 was 47.4 ng/µL DNA, and in hair follicles sample 3 was 46 ng/µL DNA, the difference was 1.4 ng/µL less in hair follicles. In blood sample 4 was 49.1 ng/µL DNA and in hair follicles sample 4 was 40.5 ng/µL, and the hair follicles DNA quantity was with 8.6 ng/µL less than blood. The sample number 6 registered in blood 54 ng/µL DNA and in hair follicles 50 ng/µL DNA. The samples for goat number 9 were with 27.7 ng/µL DNA in blood and 25 ng/µL in hair follicles. In the samples from goats 3, 4, 6, 9, and 11 were less DNA quantity in hair follicles than in blood samples, with differences between 1.4 ng/µL and 8.6 ng/µL. In the others 15 samples have been differences of DNA quantity between blood samples and hair follicles sample from 9.5 ng/µL to 29.4 ng/µL (Table 4). Even if there are less DNA quantity in hair follicles than in blood samples, the DNA amount isolated from hair follicles was sufficient for a specific DNA fragment amplification by PCR. In this study was used the dsDNA High-Sensitivity (HS) Assay Kit because it can detect DNA from 0.1 ng which are suitable for the hair follicles samples with a lower DNA quantity.

Table 4

The results of total DNA quantification of whole blood and follicle samples

#Goat	Blood ng/µL	Hair follicles ng/µL	Difference ng/µL
1	22	12.5	9.5
2	43.1	22.3	20.8
3	47.4	46	1.4
4	49.1	40.5	8.6
5	44.9	15.5	29.4
6	54	50	4
7	38.8	15.7	23.1
8	31	16.7	14.3
9	27.7	25	2.7
10	31.6	12.4	19.2
11	19.3	11.5	7.8
12	36.7	24	12.7
13	25.5	10.5	15
14	46	24	22
15	47.3	21	26.3
16	33.7	17.3	16.4
17	35.8	19.7	16.1
18	37.4	21.5	15.9
19	45.3	32.8	12.5
20	37.7	10	27.7
Sum	*754.3*	*448.9*	*305.40*
Average	*37.72*	*22.45*	*15.27*
Variance	*90.91*	*133.66*	*66.16*
STDEV	*9.54*	*11.56*	*8.13*

Anova: Single Factor, alpha=0.05

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2331.729	1	2331.729	20.76517	0.000052	4.098172
Within Groups	4267.035	38	112.2904			
Total	6598.764	39				

Suenaga and Nakamura (2005) used a commercial DNA extraction kit similar to the one used in our study, the amount of DNA isolated from the hair was less than 120 ng/µL. Zabek et al. (2005) compared different DNA methods of isolation from hair and there were obtained DNA concentration (ng/µL) from 29.5 ng/µL to 218.5 ng/µL depending the number of the follicles hair and the method used. The ethanol precipitation method having DNA concentration from 29.5 ng/µL and Proteinase K digestion method having DNA concentration from 188 ng/µL in hair follicles samples (16). The commercial kit used in our study is based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts and ethanol, digestion being made by proteinase K. Based on the previous study, the results are comparable in the case of the ethanol method 29.5 ng/µL DNA concentration from hair follicles sample in Zabek et al (2005) study and 10 - 46 ng/µL in our study. In the proteinase K method, the results from our study quantify lower DNA concentration that Zabek et al. (2005), maximum 46 ng/µL DNA concentration from hair follicles sample in our study and 218.5 ng/µL DNA concentration in Zabek et al. (2005) study. According to Guan et al. (2013), the results of quantifying the DNA from hair shafts was between range 1.25-80 ng/µL, which is similar to our results from hair follicles being between range 10-46 ng/µL. Therefore, the results from literature (1, 6, 14-16) are in accordance with our study that the DNA can be isolates from hair samples and can be quantify by the different quantitative methods.

CONCLUSIONS

The DNA amounts obtained from whole blood and follicles hair were enough for the specific amplification of 1200bp DNA fragments. In order to obtain a sufficient amount of DNA from the goat hair follicles, special attention must be paid to the number of follicles obtained. In our research, a value close to 40 proved to be the best for obtaining a quality DNA concentration. The hair follicles of goat can be used as a non-invasive method of sampling biological material for the genomic DNA isolation.

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