



Efficient, fast and low-cost strategies for DNA extraction from different nucleated sheep cells

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ABSTRACT. DNA extraction is usually the first step to perform molecular studies. This process can be nonviable due to genomic DNA (gDNA) extraction commercial kits prices. Furthermore, available DNA extraction protocols generally have high specificity, limiting their use to specific sources of biological material. In order to reduce costs, optimize time and laboratory logistics, besides to demonstrate a versatile protocol, the present study worked on an efficient DNA extraction protocol from somatic and non-somatic cells, using biological material from sheep as a model. For that, gDNA was extracted from whole blood, spermatozoa, and hair bulb cells, collected from three adult sheep, transported at 5°C and stored at -20°C until lab procedures. After extraction, gDNA concentration and purity were evaluated in a nano spectrophotometer. gDNA concentration from whole blood was greater ($p < 0.05$) than extracted from hair bulb cells, which in turn was superior ($p < 0.05$) than in spermatozoa. Also, gDNA from whole blood and, followed by, sperm showed greater ($p < 0.05$) purity when compared to gDNA of hair bulb cells. Adapting a gDNA extraction protocol, originally developed for bovine whole blood, enabled to obtain and isolate gDNA in different nucleated sheep cells.

Keywords: Biotechnology; DNA extraction; hair bulb; spermatozoa; blood; genetic improvement.

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Introduction

The advent of DNA discovery and its applications in molecular sciences made possible some advances in health, research and animal production (Ristanic et al., 2018; Shalaby et al., 2019; Yadav, Tomar, Jha, & Singh, 2017; Yamamoto et al., 2017). However, genetic resources access and use in most of developing countries is lower than expected (Food & Agriculture Organization of the United Nations (FAO, 2010). These factors reinforce the evident need to optimize biotechnological processes, as well provide basic molecular methodologies as tools for economic and social development.

In molecular biology studies, many analytical techniques use DNA (Coelho et al., 2004). Several DNA extraction methods are well established for different biological sources (Warton, Saskova, Dalihodova, & Vanek, 2018), applying manual and automated processes (Votrubova, Saskova, Dalihodova, & Vanek, 2017). Despite the wide variety of extraction protocols available, some of them involve the use of expensive reagents. Furthermore, they have high specificity. For example, it is known that DNA extraction protocols from somatic cells are not effective in non-somatic cells (Darbandi et al., 2018), factor that results in versatility reduction of available protocols for its use in different biological sources (Araújo et al., 2009; Coelho et al., 2004; Oliveira et al., 2007). Nevertheless, new DNA extraction methods can be developed or adapted, representing important strategies and optimizing existing protocols (Oliveira et al., 2007).

The importance of standardizing techniques for isolation and conservation of animal genetic resources, such as DNA, is extremely relevant for genetic diversity maintenance (Tesema & Shenkute, 2019), contributing to the preservation of native species. In this sense, sheep have an important representation in the world livestock scene, associated with production of meat (Chikwanha, Vahmani, Muchenje, Dugan, & Mapiye, 2018), milk for cheese (Martini, Scolozzi, Cecchi, Mele, & Salari, 2008), wool (Safari, Fogarty, & Gilmour, 2005) and leather. However, few studies made possible DNA extraction from species with economic

importance. Therefore, the aim of this study was to adapt and standardize a protocol for rapid DNA extraction from different nucleated sheep cells.

Material and methods

Firstly, this study used Araújo et al. (2009) methodology for genomic DNA (gDNA) extraction from bovine whole blood. To verify its effectiveness for sheep cells, gDNA extractions were performed from sheep blood cells (BC) and employed as a control treatment. Then, the same protocol was modified and adapted to extract DNA from hair bulb cells (HB) and spermatozoa (SP).

Ethical approval

All procedures were in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA). The project was carried out following the ethical aspects for the use of animals, and previously approved by the Committee on Ethics in the Use of Animals (CEUA) of the School of Veterinary Medicine of *Faculdade Pio Decimo*, with registration number 32/18.

Experimental design

Whole blood, animal hair and semen samples were collected from three male sheep (*Ovis aries*) belonging to the Rio da Mata Farm Study Center (Lat: $-11^{\circ}15'51.17''$, Long: $-37^{\circ}27'02.73''$) of *Faculdade Pio Decimo*, state of Sergipe, Brazil. The animals were raised with free access to supplemented mineral salt and water in a semi-intensive regime. Before sample collection, the sheeps showed normal clinical and body conditions, as well body temperature and ocular mucosa.

Sample collection and processing

Whole blood samples were collected through jugular venipuncture, using vacuum collection system and stored in 4 mL tubes, containing 5% anticoagulant (EDTA).

Semen samples, to obtain spermatozoa cells (SP), were collected using an electro-ejaculator (AUTOJAC V3) via rectal with the animal in the station and conscious. Before collection, symbols were removed from the rectal ampoule; the foreskin was washed with running water and mild soap, and both probe and rectum lubrication was performed with mineral oil to begin semen collection. The ejaculate, from each animal, was transferred to a 50 mL tube and, then in laboratory conditions, gDNA was extracted from different concentrations of sperm cells: 100, 200, 300 and 400 x 10⁶ spermatozoa (cells ml⁻¹), in two replicates per concentration, and then analyzed in 10 repetitions.

Hair samples, to obtain hair bulbs (HB), were collected along the animal withers where hair manifest greater length in relation to the rest of the body, facilitating their collection. The hair shafts were pressed with the aid of anatomical forceps at their base and pulled in a caudal direction. The gDNA was extracted from 30, 60, and 90 units of animal hair containing intact hair bulb and evaluated in 10 repetitions.

Therefore, whole blood, semen, and animal hair samples were transported in an isothermal box at 5°C to the laboratory and then stored at -20°C until processing.

gDNA extraction was developed in laminar flow hood. Whole blood samples were homogenized by tube inversion after thawing at room temperature; then, transferred to sterile tubes according literature (Araújo et al., 2009). In the case of seminal samples, those were thawed at room temperature and, initially, measured sperm concentration (SCon) according Silva, Nogueira, and Silva (2017). After assessing SCon, semen was diluted in aliquots of 100 x 10⁶ spermatozoa (cells ml⁻¹). To obtain SP, semen samples from the aliquots in each concentration were centrifuged at 10,000 x g for five minutes in an uncooled centrifuge and then removed the supernatant. Afterward, these semen aliquots were washed (Manuja, Manchanda, Kumar, Khanna, & Sethi, 2010) and centrifuged three times at 5,000 x g for 5 minutes in 1 mL of 1x sterile saline phosphate buffer solution (PBS), in order to purify spermatozoa from the other seminal plasma components (Darbandi et al., 2018).

Hair shafts with intact HB samples were thawed for one minute at room temperature and then submerged in a petri dish containing 100 mL solution of 2.5% sodium hypochlorite in distilled water during 5 minutes. Then they were transferred, with the aid of tweezers, to a new petri dish filled with pure distilled water (Almeida, Betancor, Fregel, Suárez, & Pestano, 2011). After the last wash, the HB were carefully collected, counted, and transferred to 2.0 mL sterile tubes.

Genomic DNA extraction

In the present study, the protocol executed by Araújo et al. (2009) for BC was reproduced. However, modifications for DNA extraction from SP and HB were applied, due their cellular composition peculiarities. Protocol characteristics are summarized in Figure 1, describing the sequence of gDNA extraction steps for SP and HB.

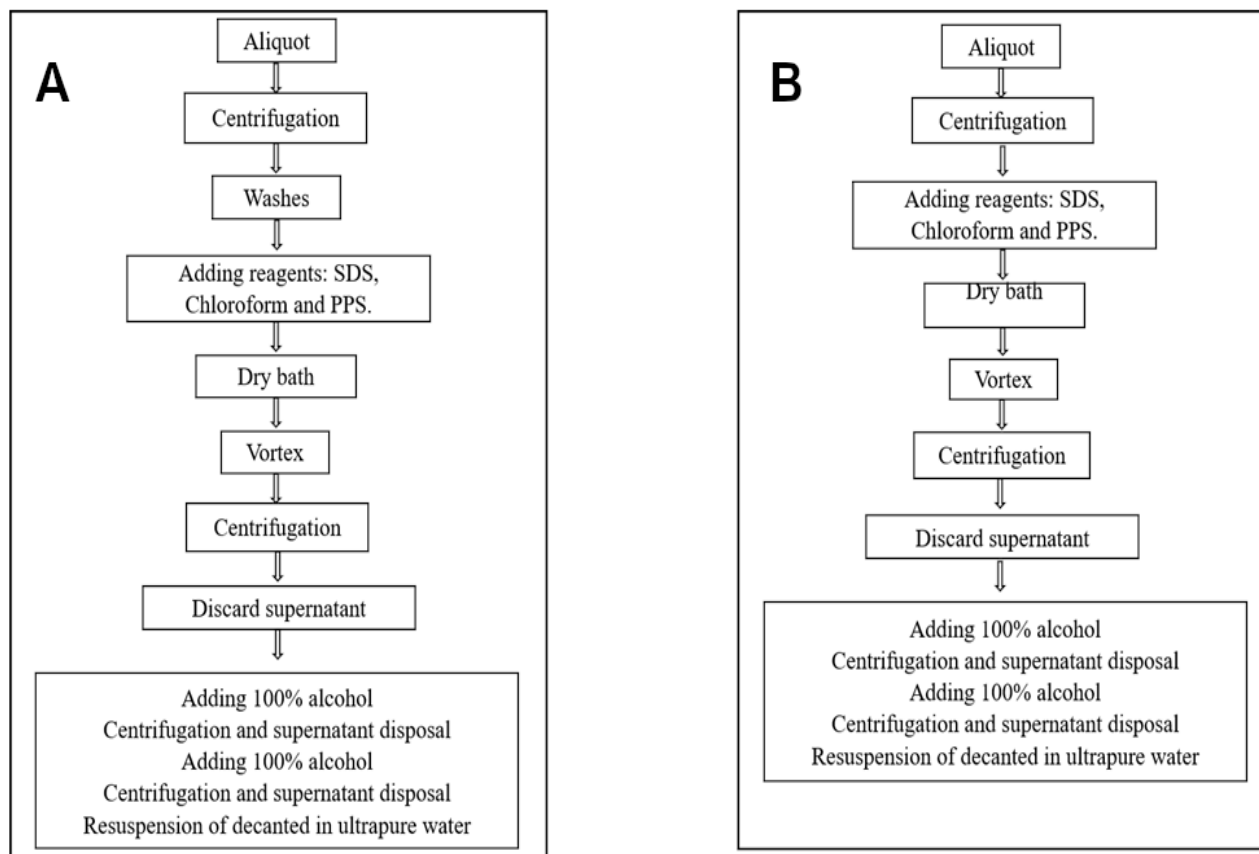


Figure 1. Schematic representation of the steps of the DNA extraction protocol adapted for spermatozoa (A) and hair bulb cells (B). PPS: Protein precipitation solution; SDS: Sodium dodecyl sulfate.

For SP, vortex homogenization time was changed after adding reagents at lysis step to three minutes, after that the exposure to dry bath for two hours at 60°C. The volume of protein precipitation solution (PPS) was changed to 500 μL , and the initial volume for aliquoting after washing was 100 μL at the different spermatozoa counts to be evaluated.

For HB, vortex homogenization was modified to two minutes. The protein precipitation solution volume and the temperature of exposure to the dry bath were the same for SP.

After extraction, for BC, SP and HB, gDNA pellet was resuspended in 100 μL of ultrapure water and slightly homogenized through five minutes for solubilization. Finally, solubilized gDNA samples were stored at -20°C.

DNA concentration and purity assessment

Both gDNA purity and concentration were measured with the aid of a nano spectrophotometer (Thermo Scientific NanoDrop Lite). For this, 2 μL of the suspension containing gDNA from each sample was used. All aliquots of gDNA extracted from BC, SP, and HB were measured in ten repetitions. gDNA purity is estimated from the A260/A280 absorbance ratio, where gDNA absorbs light at a wavelength of 260 nm whereas proteins at 280 nm. The gDNA concentration ($\text{ng } \mu\text{L}^{-1}$) is expressed by the quantitative measure of light absorption by the solutions, in which the substance is proportional to the amount of light absorbed (Desjardins & Conklin, 2011).

Agarose gel electrophoresis

gDNA samples from sheep BC were submitted to electrophoresis for evaluation on 1% agarose gel in 1% Tris Borate EDTA (TBE), as experimental control. For comparison purposes, a well of the agarose gel was assigned to the Ladder (7 μL of Ladder + 5 μL of Diamond Nucleic Acid Dye + 5 μL of Coomassie Blue).

DNA concentration and purity assessment

To estimate differences in gDNA purity and concentration, ANOVA test was used with the following mixed linear additive model $Y_{ijk} = \mu + T_i + R_j + E_{ijk}$. Where: Y_{ijk} is value of the dependent variable for concentration and purity, μ represents the average of the total population expected, based on the Y_{ijk} observation. T_i represents the fixed effect of the i -th tissue studied, R_j indicates the random variable indicated for each repetition associated with the measurement of Y_{ijk} and, finally, E_{ijk} represents the residual random effect associated with each observation. Comparisons of means among different cell groups was applied when significant effect ($p < 0.05$) between tissues was observed, using Tukey test with 5% statistical confidence. All analyzes were performed using the statistical software R (R Core Team, 2020).

Results and discussion

This study presents a rapid and low cost evaluated protocol of extraction and isolation of DNA from different biological materials of sheep, such as BC, SP, and HB. The development of novel DNA extraction strategies is important to implement molecular biology routines for the assessment of genetic markers, which is relevant in order to increase production. The adapted protocol provided high concentrations of DNA extracted in the control group that used sheep BC, as evidenced in the electrophoresis gel (Figure 2). The protocol used in the present research showed high extraction efficiency for intact gDNA in terms of concentration.

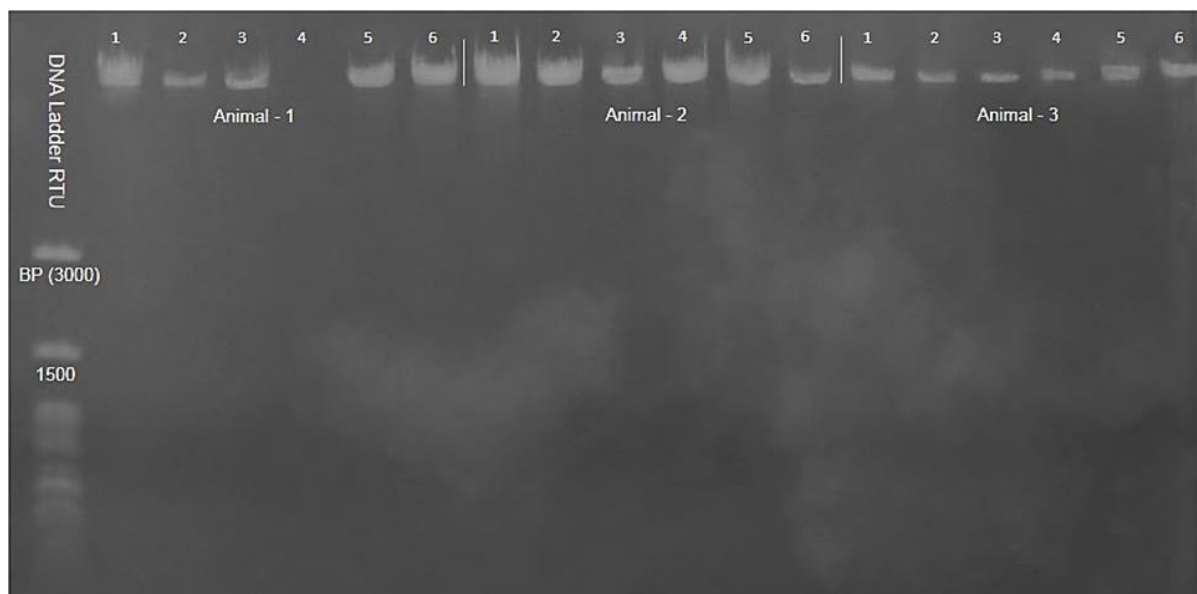


Figure 2. Agarose electrophoresis gel with blood samples from three sheep. In the first well, DNA Ladder RTU. Following, six replicates (1-6) per animal were performed for each sample.

BP: base pair.

The protocol executed and then adapted in this study lasted an average of 40, 180, and 160 minutes for BC, SP and HB cells, respectively. Following extraction steps processes, the protocol efficiency was measured by ten biological samples simultaneously. The study also enabled DNA extraction from SP and HB cells.

Table 1 shows both concentration ($\text{ng } \mu\text{L}^{-1}$) and purity (A260/A280) of gDNA in the present research. gDNA from BC had the highest concentration and purity, while HB obtained higher concentration ($p < 0.05$) when compared to SP. The purity, however, was higher ($p < 0.05$) in SP compared to HB.

Table 1. Mean \pm standard error of the concentration ($\text{ng } \mu\text{L}^{-1}$) and purity (A260/A280) of gDNA obtained from blood cells (BC), spermatozoa (SP) and hair bulb cells (HB) from sheep.

Treatment	Concentration ($\text{ng } \mu\text{L}^{-1}$)	Purity (A260/A280)
BC	79.258 ± 1.715 a	1.656 ± 0.009 a
SP	2.0381 ± 0.220 c	1.350 ± 0.081 b
HB	10.497 ± 1.919 b	0.977 ± 0.051 c

$\text{ng } \mu\text{L}^{-1}$: nanograms per microliter; A260/A280: absorbance ratio of the isolated DNA. Means \pm standard error values followed by different letters in the same column differ statistically ($p < 0.05$).

Concentration ($\text{ng } \mu\text{L}^{-1}$) and purity (A260/A280) from SP and HB gDNA are shown in Table 2. When comparing gDNA quality from different spermatozoa counts, it was observed that the gDNA concentration was higher ($p < 0.05$) when the highest (400×10^6 spermatozoa cells mL^{-1}) spermatozoa was used. Through the use of spectrophotometry, it was not possible to detect isolated DNA when 100 million spermatozoa were evaluated, and lower concentration was obtained ($p < 0.05$) when 200 million spermatozoa were used. The gDNA purity were similar ($p > 0.05$) when 300 or 400 million spermatozoa were evaluated.

In relation to gDNA extracted from HB cells, the highest concentration of gDNA ($p < 0.05$) was obtained when 90 hair shafts units were used. Using 30 hair shafts units was responsible for the lowest concentration gDNA ($p < 0.05$). The gDNA quality, in turn, was similar ($p > 0.05$) among the different hair shafts units.

Table 2. Mean \pm standard error of concentration ($\text{ng } \mu\text{L}^{-1}$) and purity (A260/A280) of gDNA obtained from under different spermatozoa counts (SPcount) and hair bulb units (HBunits).

Nucleated cells		Concentration ($\text{ng } \mu\text{L}^{-1}$)	Purity (A260/A280)
SP count $\times 10^6$	100	/	/
	200	0.970 ± 0.021 c	1.485 ± 0.045 b
	300	2.480 ± 0.110 b	1.651 ± 0.035 ab
	400	4.570 ± 0.037 a	1.659 ± 0.014 a
HB units	30	0.120 ± 0.013 c	0.775 ± 0.133 a
	60	6.780 ± 0.073 b	1.025 ± 0.004 a
	90	24.590 ± 0.114 a	1.130 ± 0.004 a

$\text{ng } \mu\text{L}^{-1}$: nanograms per microliter; A260/A280: absorbance ratio of the isolated DNA. /: No data recorded. Means \pm standard error values followed by different letters in the same column differ statistically ($p < 0.05$).

The improvement and adaptations applied in the protocols proposed in this work were based on DNA extraction methods Salman and Laureano (2006); Almeida, Alencar, Barbosa, Dias, and Almeida (2013) and Guan et al. (2013) which used similar biological materials, temperatures and dry bath exposure times, but with different DNA extraction protocols. The studies of Manuja et al. (2010), Silva et al. (2014), Wu, De Gannes, Luchetti, and Pilsner (2015), and Darbandi et al. (2018) also served as basis for procedures developed in the current research.

DNA extraction from BC presented the highest concentration, which can be attributed to the higher number of nucleated cells (leukocytes) in the whole blood. Crispim et al. (2016) and Caldart, Chiappetta, Lopes, and Ravazzolo (2011) demonstrated the relationship between the concentration of extracted DNA and the number of nucleated cells when extracting DNA from leukocyte.

In another study, Araújo et al. (2009) evaluated the effectiveness of a DNA extraction protocol for bovine whole blood in the whole blood of buffaloes (*Bubalus bubalis*) and red deer (*Ozotoceros bezoarticus*). The average concentration obtained were 24.36 and 73.72 $\text{ng } \mu\text{L}^{-1}$, respectively. It was observed in the present study that the behavior of this protocol as control treatment showed better results when compared to the extraction of DNA from blood cells from the buffalo and cervid species. Variations in the concentration of DNA extracted from the same protocol, in different species, may be related to the divergent leukocyte profile between them (Araújo et al., 2009; Ristanic et al., 2018), since the protocol acts on nucleated cells isolated from the whole blood, which may suffer variations due to external factors, such as stress, causing profile differences among individuals of the same species, albeit in an abnormal way.

Caldart et al. (2011) evaluated two commercial kits (DNAzol[®] - Invitrogen[™], FTA[®] - Whatman[™]) for DNA extraction for goat and sheep leukocyte rings as a function of storage time. The concentrations ($\text{ng } \mu\text{L}^{-1}$) obtained from whole blood in this study (79.258 ± 1.715) were numerically higher than those obtained by DNAzol[®] kit. Likewise the purity (1.66 ± 0.009) here obtained, was greater than that achieved using FTA[®] kit. The differences observed between the extraction methods may be related to DNA storage, since the method used in the present study indicates evaluations in shorter intervals after extraction. The extraction protocol of the present research proved to be satisfactory for sheep BC, when compared to the study that used leukocyte ring, revealing the efficiency of the reagents used in relation to commercial kits, regarding the concentration and purity obtained.

The results presented here were superior to studies that used commercial kits regarding the concentration of DNA extracted from blood cells (Riemann et al., 2007). This result is quite relevant since the acquisition of commercial kits, despite resulting in DNA extractions sometimes in high concentration and good quality, demand high investment costs (Araújo et al., 2009).

For SP gDNA extracting procedure, Manuja et al. (2010) qualitatively evaluated protocols from Buffalo semen (*Bubalus bubalis*), in which it was observed that the protocol involved the use of

cetyltrimethylammonium bromide (CTAB) and phenol-chloroform reagents, obtaining DNA purity numerically superior to those obtained in the present work. In the same study, when they used TRIzol® (Sigma™) the purity was approximately of 1.7, similar value than the obtained in the present study, which is characterized as low-cost protocol in relation to commercial DNA extraction kits.

The increase in purity in DNA extraction observed by Manuja et al. (2010) when implementing phenol-chloroform may be a result of the action of phenol. According to Oliveira et al. (2007), this reagent causes efficient protein denaturation. Regarding purity, CTAB method is widely used in which DNA is mixed with detergent and, later, separated by isopropanol, presenting high versatility when used. Although the reagents mentioned were not tested in the standardization of the present protocol adaptation, which would increase its complexity, duration, and cost, our results still able to provide consistent DNA purity. The other protocols in Oliveira et al. (2007) study, consisted of using commercial kits such as Chelex-100 resin® (Sigma™) and QIAamp® DNA mini kit (Qiagen™) added with proteinase K, which can make the application of the protocol more efficient, but more expensive.

Still related to the DNA extraction procedure that used SP, Silva et al. (2014) evaluated three protocols using goat semen, obtaining interesting values as 2.0 (DNeasy Blood and Tissue Kit - Qiagen™); 1.63 (phenol - chloroform) and 0.98 (Chelex-100® resin - Sigma™). Those values were lower than those observed in this study, considering that values above 2.0 indicate contamination, usually by RNA. As for the concentration, values above 100 ng μL^{-1} may be related to the use of proteinase K, a highly effective recombinant enzyme to promote protein denaturation and nucleic acid exposure.

The concentration of DNA extracted in SP was lower than the other treatments in this study. Different authors emphasize the existence of protamines, spermatozoa DNA binding proteins that are responsible for the compaction and greater rigidity of the nucleus in these cells. This exclusive protein organization of spermatozoa hinders the action of detergents, salts and reducing agents used in the protocol in order to obtain DNA samples in high concentrations (Silva, Arruda, Oliveira, Nascimento, & Moura, 2008; Silva et al., 2014; Wu et al., 2015).

Despite this, SP results in the current research are satisfactory, since they have high degree of purity. Although this parameter does not affect DNA viability, such as concentration, which was lower than that found by Darbandi et al. (2018), that used TRIzol®, proteinase K and chloroform in association to process human semen. However, it is important to consider the increased complexity of the procedure, with the involvement of several additional steps, which affect the practicality and exponentially increase the cost of the process.

A cost survey applied in market found that 1 g of lyophilized proteinase K costs more than the total sum of the volume of all reagents used in the adapted protocol used in this study, evidencing the low-cost character of this protocol.

In addition, the results here presented, considering SP, were different from other studies evaluating the extraction of DNA from an established number of SP in a solution. Studies generally consider volume, rather than the concentration of cells in the ejaculate (Anvar, Namavar-Jahromi, Ebrahimi, & Gharesi-Fard, 2015; Draškovič, 2017; Manuja et al., 2010; Silva et al., 2014). The DNA concentration extracted from spermatozoa allows the genetic resources optimization use, increasing semen collection intervals, and the use of a single ejaculate for the application of different approaches. Moreover, the results of this study show that the extraction protocol used was effective in both somatic (BC and HB) and non-somatic (SP) cells, despite what has already been reported that protocols for gDNA extraction in human somatic cells are not efficient for non-somatic cells, such as spermatozoa (Darbandi et al., 2018).

For HB gDNA extraction procedure, it was possible to evidence a dependent relationship between the number of hair shafts containing intact bulb and the increase in the concentration of gDNA. The constant in the variation of concentration may be related to the greater number of HB, since Salman and Laureano (2006) mention the importance of these for gDNA extraction, being the place where the greatest number of nucleated cells is found.

Pfeiffer, Völkel, Täubert, and Brenig (2004), studying the DNA extraction from canine hair achieved, on average, DNA concentration of 32.04 ng μL^{-1} from 100 hair units. These values are similar to those found in the current study when 90 hair units were used. In addition, the same author used dry bath incubation time of more than two hours in their protocol, using proteinase K in the lysis buffer. This demonstrates the high efficiency of the protocol used in the present study since a shorter incubation time was used, and still without the use of proteinase K.

Still considering the procedure for HB, Salman and Laureano (2006) evaluating three genomic DNA extraction protocols, from 5 and 40 by those from the bovine withers, found the best result in the trial that used proteinase K, phenol - chloroform and 40 hair units with a purity of 1.8 and a concentration of 753 ng μL^{-1} . However, this protocol proved to be expensive and laborious, lasting 28 hours.

In another study, Guan et al. (2013) evaluated three enzymatic methods of extracting gDNA from goat hair using enzymatic laundry powder from different brands. The concentration averages obtained using soap were lower than those obtained in this study, with 15.82 ng μL^{-1} being the best concentration obtained with Diao™.

The use of automated extraction protocols, such as PrepFiler BTA™ and AutoMate Express™ for isolating genomic DNA from human hair, used by Almeida et al. (2011), are characterized by high cost, being justified for forensic approaches that use sources with scarce amounts of biological material in solving crimes, for example. Hence, due to their cost, they can hardly be applicable in animal science.

DNA extraction from HB cells proved to be the least invasive and practical method from the point of view of material collection, despite providing samples with lower purity compared to other treatments. Hair shafts have a high concentration of keratin that degrades DNA. Hence, they have melanin and eumelanin along the entire stem, known as inhibitors of the PCR reaction (Bessetti, 2007), factor than can influence the purpose of the DNA extracted.

Conclusion

The present study promoted to extract DNA through a simple methodology, using low-cost reagents for spermatozoa and hair bulb cells. In addition, it was very efficient in sheep whole blood, with adequate concentration and purity for other molecular procedures. DNA extraction from whole blood, spermatozoa, and hair bulb cells proved to be fast and with good performance, enabling to optimize molecular biology laboratory routines. In order to improve extracted DNA purity from spermatozoa and hair bulb cells using this protocol, further studies are needed; since it represents a non-invasive, non-painful and practical alternative for DNA extraction.

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