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Research Article

COMPARATIVE ASSESSMENT OF FIVE DISTINCT DNA EXTRACTION PROTOCOLS FROM FROZEN BUFFALO SEMEN

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ABSTRACT: Selection utilizing genetic markers is a key aspect in achieving genetic improvement in populations. Genomic and proteomic analyses require a sufficient quantity of good-quality DNA, which can be isolated from animal sperm, hair, blood, tissues, saliva and cartilage. Due to the presence of solvents and dilutions in frozen semen, the physical and chemical properties of sperms and the non-cellular percentage of ejaculate, DNA extracted is often of poor quality. As per the literature, a wide variety of DNA extraction methods from semen was available. In the present study, five different extraction procedures were adopted to assess the quality and amount of DNA extracted from frozen semen straws. All the methods were found to obtain DNA, which was verified by nanodrop readings; however, the genomic DNA obtained by method I could not be visualized on 1% agarose gel. The DNA quality and quantity, obtained from these methods differ significantly ($p<0.01$). Successful amplification of the target sequence by PCR was observed with DNA extracted using the III and V methods. In conclusion, method V (modified high salt method) is the best procedure to obtain good quality and quantity DNA from buffalo frozen semen straws without any constraints in PCR.

Keywords: Frozen semen, Guanidium, DNA, Isolation, PCR.

INTRODUCTION

To enhance milk production in terms of quality and quantity, disease resistance, draught capacity, number of lactations, and so on, superior bulls and cows must be selected using genetic markers. Polymerase Chain Reaction (PCR) is the first step in the analysis of genetic markers and their incorporation into the population [13]. PCR is a relatively quick, sensitive, and simple technique that allows for the quick amplification and in vitro examination of specific DNA sequences. The DNA template, quantity, and quality are necessary for the PCR process to be effective [14, 21]. Due to extensive artificial insemination programs, semen is a frequent source of the transfer of viral, bacterial, and parasite infections to cattle and buffaloes [7]. Therefore, a feasible strategy for reducing the prevalence of these illnesses is to screen the frozen semen straws for the DNA of disease causing agents regularly. There are several common techniques for isolating DNA from various tissues; however isolating DNA from semen is a difficult process since seminal plasma contains fructose, proteins, and minerals like zinc and copper that reduce the quality of the results [14].

Furthermore, the disulfide linkages in the spermatozoa plasma membrane make them more difficult to lyse [4, 20]. Additionally, the diluents used to store frozen semen contain lipids, proteins, and minerals, thus it is required to completely remove them to extract DNA of high quality [9, 16]. An attempt was made to evaluate the quality and quantity of DNA produced by each of the five widely used DNA extraction methods of semen's DNA isolation protocols.

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MATERIALS AND METHODS

The chemicals in this study were all purchased from Himedia, Mumbai, India, and none of the reagents used in the experiment have expired. All the consumables, including the glassware and plastic ware utilized in this investigation, were purchased from Tarson, Kolkata, and Borosil, Mumbai, respectively. The oligonucleotide primers in the study were custom synthesized from Bioserve Biotechnologies in Hyderabad, India.

Semen samples

Forty frozen semen straws were taken from a single Murrah bull, at the frozen semen bull station, in Visakhapatnam. They were transported to the lab in liquid nitrogen. The semen straws were thawed for 30 seconds at 37°C before using them for DNA isolation. For each technique, four samples were isolated.

DNA extraction from frozen semen

The DNA was extracted from the thawed semen using the following five methods.

Method I

Thawed semen, amounting to 300 µl, was taken into a microcentrifuge tube of 2 ml volume. Subsequently, 200 µl of Lysis Buffer-I (4M Guanidium thiocyanate, 0.1M Sodium acetate, 0.4 mg/ml Proteinase K, and 5% Glycerol, pH-7) was added, thoroughly mixed, and left to incubate at 24 °C for 5 minutes. Following this, 40 µl of chloroform was added and mixed with it for 15 minutes, and then centrifugation was done at 12,000 Xg (15 minutes at 4°C). The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube, avoiding disturbance of the interphase. DNA precipitation was achieved by washing with 100 µl of ice-cold saturated ethanol, followed by centrifugation at 12,000 Xg at 4°C for 10 minutes. After that discard the supernatant and the pellet was washed with pre-chilled 75% ethanol and centrifuged for 5 minutes at 12,000 Xg and 4 °C. The resulting pellet was air-dried, reconstituted in nuclease-free water, and incubated at 56 °C for 20 minutes.

Method II

The 300 µl of thawed semen was added to 1 ml of PBS (137 mM NaCl, 2.7mM KCl, 10 mM $K_2H_2PO_4$, and 10 mM Na_2HPO_4; pH-7.4) and centrifuged at 5000 rpm for 10 minutes. This procedure was repeated for 2 times and to the pellet 1 ml of Lysis Buffer-I was added and incubated for 4 hours at 55 °C. To the supernatant 1ml of 25:24:1 phenol: chloroform: isoamyl alcohol (P:C:I) was added and mixed for 10 minutes, further it was centrifuged (13,000 rpm and 4 °C) for 10 minutes. The aqueous layer present at the top was removed and 0.1 volume of 3M sodium acetate and isopropanol of equal amount was added and mixed gently. After incubating the mixture at - 20 °C overnight, centrifugation was done at 13000 rpm for 10 minutes at 24 °C. To the pellet addition of 500 µl, 70 % ethanol, and centrifugation at 13000 rpm for 10 minutes was done. The nuclease-free water was used for re-suspension after air drying.

Method III

The prewashing of the 300 µl of thawed semen with PBS, pH 7.4 was done similarly to the above procedure. To the pellet obtained, 1 ml of Lysis buffer-II (1M Tris HCl, 0.5 M EDTA, 5 M NaCl, and 4% SDS) and 4 µl of proteinase K was added and then mixed thoroughly by gentle vortexing which was incubated at 56°C for 1 hour. To this 1 ml of saturated 25:24:1 P:C:I mixture was added, mixed, and centrifuged for 10 minutes at 13000 rpm and 24 °C. To this equal proportion of chilled isopropanol was added along the walls and centrifuged for 10 minutes at 13,000 rpm. After discarding the supernatant 1ml 70% ethanol was added to the pellet and centrifugation was done at 13,000 rpm for 5 minutes. The nuclease-free water was used for re-suspension of the pellet and incubated at 56 °C for 20 minutes after air drying.

Method IV

To the pellet which was obtained after pre-washing with PBS, pH 7.4 for 3 times as first method, 1 ml Lysis buffer and 4 µl of proteinase K were added and incubated at 56°C for 4 hours after thorough mixing. To this, 24:1 Chloroform: Iso-amyl alcohol (C: I) was added and mixed well, after which centrifugation was done for 10 minutes at 24°C and 13,000 rpm. Separation of the upper layer into the new tube was done to which 90 µl of sodium acetate and 900 µl of isopropanol were added and mixed gently, centrifugation was done at 13000 rpm for 10 minutes, and to the pellet, we added 500 µl of ice-cold 70% ethanol and centrifuged at 2000 rpm for 5 minutes. The pellet resuspension was done similarly to the previous procedure.

Method V

Add 500 µl of Lysis buffer-III (50 mM Tris HCl, 20 mM EDTA, 10% SDS; pH 8) to the pre-washed

semen with PBS, pH 7.4 as the first method. To this, we added 10 µl of proteinase K and incubated at 56 °C overnight. To this mixture 50 µl of 2M sodium chloride was added and mixed for 10 minutes. Further, 20 µl of 24:1 of C:I mixture was added and mixed for 5 minutes after which centrifugation was done for 15 minutes at 13000 rpm. To the upper aqueous layer, 900 µl of ice-cold absolute ethanol was added in another tube and centrifugation was done for 10 minutes at 13000 rpm. To this pellet, 200 µl of 70% ethanol was added and centrifugation was done for 5 minutes at 2000 rpm. The air-dried pellet was resuspended similar to the previous procedure.

Quality and quantity of DNA

Gel electrophoresis of 1 % concentration was used to confirm the genomic DNA, which was prepared by boiling agarose in 0.5X TAE (Tris Acetate-EDTA) buffer [12]. To the agarose, ethidium bromide was added at the rate of 0.5µg/ml. The prepared gel was added to an electrophoresis plate and allowed to polymerize for around 30 minutes at room temperature. One µl of 6X loading buffer containing bromophenol blue dye and six µl of dH_2O were mixed and to this 2µl of DNA sample was added and then loaded into the wells of the gel. The DNA samples were run on agarose gel at 100 V for around 60 minutes in 0.5X TAE buffer and the gel was visualized under a gel image system (Omega Fluor™ Plus Documentation Systems, BioExpress, USA).

Using a Nano Spectrophotometer from Thermo Fisher Scientific, DNA concentration was quantified using the convention that 1 optical absorbance unit at 260 nm will be equal to 40 µg per ml. DNA purity was assessed using the OD ratio at 260:280 and 260:230.

Polymerase chain reaction

Amplification of genomic DNA was carried out using primers specific to the Deleted In Azoospermia Like (DAZL) gene 3' UTR region (forward primer: GGGCACTTTCAAATTCTGAGG, reverse primer: CCTTGGAAGGAAAGGGTAGC) [11]. The PCR reaction volume is 10 µl containing 5 µl PCR master mix (2X Taq Master Mix Red, Amplicon, Denmark), 5 pmol of each primer, and 1µl of the template. All the DNA samples were amplified using the following cyclic conditions, initial denaturation at 94°C/3 min, followed by 35 cycles: final denaturation at 94°C/30s, annealing at 55°C/45s, initial extension at 72°C/30s, and a final extension at 72°C/10 min. Electrophoresis of PCR samples on 2% agarose gel [12] and visualization in a gel imaging system (Omega FluorTM Plus Documentation Systems, BioExpress, USA) were used to assess the success of the PCR from the five extraction techniques.

Statistical analysis

ANOVA was used to evaluate the DNA concentration and purity values, and differences were compared using the post hoc Duncan's test at a 0.05% significance level using SPSS software base 26 [5]. This approach was adopted as the difference between the means of DNA concentration was more [3]. The data was presented as means ± SD.

RESULTS AND DISCUSSION

To obtain an effective DNA extraction protocol, it is important to consider the following critical factors affordability, practicality, rapidity, and safety ensuring, that the process is not only efficient but also accessible to researchers [2]. Moreover, the quantity and quality of the extracted DNA are important in PCR-based assays where the success of amplification depends on the availability of sufficient, intact DNA templates. Minimizing DNA fragmentation during the extraction process is a must for preserving the integrity of the genetic material, thereby enhancing the success of PCR amplification and downstream analysis [22]. In the present study, method V yielded the highest DNA concentration (49.60 ± 7.85) and quality (A260/A280: 1.61 ± 0.004), suggesting its superior performance compared to the other methods (Table 1). The ANOVA revealed highly significant differences between the concentration of samples of different methods and between the quality of DNA (A260/A280 and A260/A230) of different methods ($p<0.01$). The differences noticed among the different DNA extraction methods are attributed to various factors, including the choice of reagents, extraction protocols, and purification steps. Method I produced a high A260/A280 ratio and a low A260/A230 ratio reflecting the contamination of the sample with RNA and guanidium respectively [17] (Table 1). Method V, which produced the highest DNA concentration and quality, may have benefitted from prolonged exposure to proteinase K, a key enzyme used in DNA extraction to degrade proteins and remove protein contaminants effectively [15].

Gel electrophoresis results (Fig. 1) corroborate the findings of the spectrophotometric analysis (A260/A230), with genomic DNA extracted using methods III and V exhibiting clear bands on the agarose gel,

Table 1. Quality and quantity of DNA obtained from different isolation protocols of semen (Mean±SD).

DNA isolation protocols	Sample size	Concentration (ng/µl)**	260/280**	260/230**
Method 1	4	24.31±2.83[ab]	11.42±0.81[b]	0.02±0.003[a]
Method 2	4	24.21±5.00[ab]	1.13±0.05[a]	0.15±0.02[b]
Method 3	4	28.53±5.70[bc]	0.80±0.02[a]	0.31±0.029[c]
Method 4	4	3.55±0.62[a]	1.61±0.20[a]	0.05±0.012[a]
Method 5	4	49.60±7.85[c]	1.61±0.004[a]	0.64±0.01[d]

** Indicates highly significant difference between methods at 1% level of significance. Column with different superscripts indicates significant difference between each other at 5% level of significance.

Table 2. The quality of isolated DNA based on genomic DNA and PCR product bands across different DNA isolation procedures.

DNA isolation methods	1% Gel (Genomic DNA)	2% Gel (PCR product)	Discussion
Method I	No visible bands	Not visible	High A_{260}/A_{280} ratio (RNA contamination) Low A_{260}/A_{230} ratio (Guanidine contamination) [17]
Method II	No visible bands	Thin bands	Thin bands during PCR (P: C: I and Guanidine inhibiting PCR) [2, 8, 19]; Increased purity (A_{260}/A_{280} ratio) (PBS) [1]
Method III	Thin bands	Clear and Distinct bands	Clear and distinct bands in PCR (without PCR inhibitors) [2, 19]
Method IV	No visible bands	Thin bands	Increased quality (A_{260}/A_{280} ratio) (PBS and C: I) [1] Thin bands during PCR (P: C: I and Guanidine inhibiting PCR) [2, 8, 19]
Method V	Clear bands	Clear and Distinct bands	High-quality and High-quantity DNA (increased exposure time to proteinase K) [15].

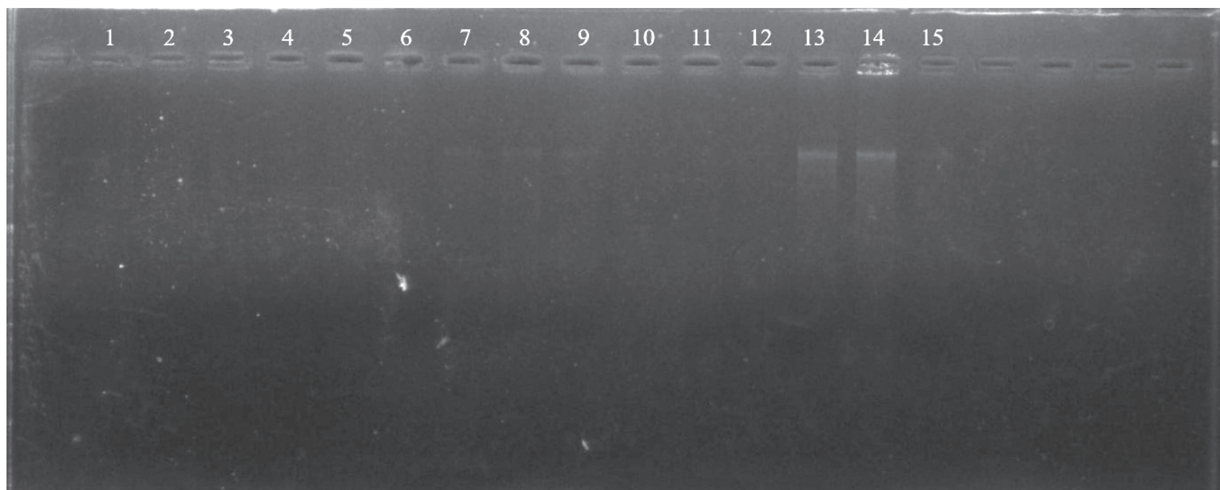

Fig. 1. Quality check of genomic DNA obtained from different methods on 1% agarose. [1, 2, 3: Method 1; 4, 5, 6: Method 2; 7, 8, 9: Method 3; 10, 11, 12: Method 4; 13, 14, 15: Method 5].

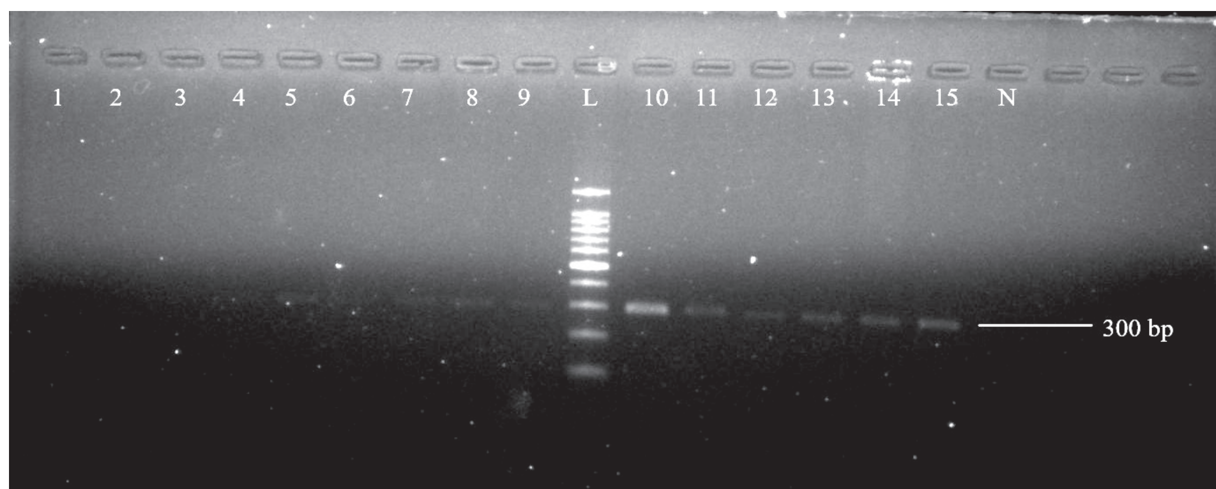

Fig. 2. PCR amplification products of DAZL/3' UTR gene in samples obtained from different methods. [1, 2, 3: Method 1; 4, 5, 6: Method 4; 7, 8, 9: Method 2; 10, 11, 12: Method 3; 13, 14, 15: Method 5; L: 100 bp Ladder; N: Negative].

indicative of intact DNA. In contrast, DNA samples extracted using other methods did not produce visible bands, suggesting potential degradation or impurities in those samples. The usage of guanidium and P: C: I in the DNA extraction process in methods I, II and IV may contribute to the DNA degradation [1, 10, 14] in the present study (Table 2). PCR amplification results (Fig. 2) further support the superiority of methods III and V in yielding high-quality DNA suitable for downstream applications. The successful amplification of the 300 bp fragment of the DAZL gene 3' UTR region from DNA samples extracted using these methods demonstrates their effectiveness in preserving DNA integrity and purity. PCR amplification of samples extracted from Method II and IV produced light bands on 2% agarose gel (Fig. 2) which may be due to usage of P: C: I [19] and guanidium which inhibits the PCR [2]. Furthermore, the genomic DNA was not amplified in method I during PCR, even though the concentration is there owing to the contamination of the sample with RNA and guanidium [6] (Table 2). A significant ($p<0.01$) increase in DNA purity in method II and method IV was observed which may be due to washing with phosphate buffer solution [1] and usage of C: I where the low molecular weight of proteins was removed respectively. P: C: I was not employed in method V because phenol-chloroform extraction is a time-consuming and potentially dangerous procedure [18], where a large quantity of DNA can be lost and degraded [10], and the PCR can be hindered [8]. In conclusion, method V is the most affordable, straightforward, and efficient approach, followed by method III, and is therefore a promising tool for extracting DNA from semen samples without any constraint.

CONCLUSION

Five methods of DNA isolation from semen straws were compared with one another in terms of quality and quantity of DNA and among them modified high salt method (method V) is the best technique for obtaining high quality and quantity DNA from frozen buffalo semen straws without any limitations in PCR.

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