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Comparative Evaluation of Five Different Methods For DNA Extraction from Semen of Buffalo Bull

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ABSTRACT

Success of PCR based molecular method is highly dependent on quality extraction of DNA template. There are many methods currently available for DNA purification from semen; but, problems related to contamination with foreign DNA, PCR inhibitors, and susceptibility to fragmentation are very common. In the present study, five different DNA extraction procedures were examined to ascertain their relative effectiveness for extracting DNA from semen samples of buffalo bull. The five methods included- two commercially supplied kits (Qiagen DNeasy Blood and Tissue Kit, Purelink Invitrogen), modified Qiagen DNeasy Blood and Tissue Kit method, Chelex 100 method and Phenol-chloroform with modified lysis buffer method. The quality and quantity assessment of the differentially extracted DNA revealed significant differences among the five procedures. Modified Qiagen DNeasy Blood and Tissue Kit method was found to be most appropriate for extracting high quality and suitable quantity of DNA from semen of buffalo bull.

KEYWORD

DNA extraction, semen, buffalo bull, evaluation

INTRODUCTION

Isolation of intact, double stranded, highly concentrated, uncontaminated genomic DNA from semen is prerequisite for success of PCR based molecular methods e.g. determination of sex ratios in (Fluoroscence activated cell sorting) FACS sorted semen samples, elucidating the role of sperm genetics and epigenetic on reproductive success etc (Grom *et al.*, 2006; Kobayashi *et al.*, 2007; Resende *et al.*, 2009; Sharifzadeh *et al.*, 2011). Unlike the somatic cells, sperm DNA is very compact due to replacement of histones with protamines. Disulphide bridges formed within and between the protamines inhibit the extraction of sperm DNA through standard techniques used for the somatic cells (Hossain *et al.*, 1997; Griffin, 2013). In addition to this, the spermatozoa are protected by a membrane which is rich in disulphide bonds, making the process of cell lysis very difficult. Although, the selection of an appropriate DNA extraction method plays a pivotal role in the success of PCR based studies, there are only a few studies in the literature that compare different extraction protocols for their relative effectiveness from semen of *Bubalus Bubalis* quantitatively and qualitatively. Thus, the aim of this study was to evaluate five methods of DNA extraction from semen of *Bubalus Bubalis* bull.

MATERIALS AND METHODS

Collection of semen samples

Semen samples were collected five times from five mature elite bulls of Murrah buffalo on alternate days, using artificial vagina method. Ejaculate from each bull was pooled at each collection time to eliminate individual variation, producing a total of five pooled semen samples, which was aliquoted into microcentrifuge tubes and stored at -20°C for further use.

Extraction of DNA from semen samples

Five different DNA extraction methods (in five replicates were used for extraction of DNA from pooled semen samples of Murrah buffalo bull. The five methods included- two commercially supplied kits (Qiagen DNeasy Blood and Tissue Kit, Purelink Invitrogen), modified Qiagen DNeasy Blood and Tissue Kit method, Chelex 100 method and Phenol-chloroform with modified lysis buffer method.

Method 1 (Qiagen DNeasy Blood and Tissue Kit)

DNA was extracted from 200 µl semen using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) as per manufacturer's instructions, and the obtained DNA was stored at -20°C for further use.

Method 2 (Purelink Invitrogen Kit Protocol)

DNA was extracted from 200 µl semen using the Purelink DNA Mini Kit (Invitrogen, Germany), according to manufacturer recommendations, and the obtained DNA was stored at -20°C for further use.

Method 3 (Modified Qiagen DNeasy Blood and Tissue Kit Protocol)

Before proceeding to the Qiagen protocol, semen was treated with two additional buffers and proteinase K. Briefly, 200 µl of semen and 10 ml of lysis buffer (150 mM NaCl and 10 mM EDTA, pH 8.) were mixed and centrifuged at 2500 x g for 10 minutes. The pellet was resuspended in 300 µl buffer containing 100 mM Tris-Cl, pH 8.0, 10 mM EDTA, 500 mM NaCl, 1% SDS and 2% 2-mercaptoethanol and then

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100 µl of proteinase K was added in it. After incubation at 56°C for 2 h, another 20 µl proteinase K was added and incubated again at 56°C for 2 h. After addition of lysis buffer and ethanol in 400 µl quantities each, the mixture was applied to the mini spin column (Qiagen, Germany) and processed according to manufacturer recommendations.

Method 4 (Chelex 100 method)

DNA from semen samples was extracted using the Chelex-100 method as described by Manuja *et al.* (2010). Breifly, 250µl semen sample aliquot was added to 200 µl 5% Chelex-100, with the subsequent addition of 5 µl proteinase K and 31 mM DTT. The mixture was vortexed, incubated at 56°C for 45 min, and then boiled in a water bath for 8 minutes to inactivate proteinase K. After vigorous vortexing for 10 s, the sample was centrifuged at 10,444 rpm for 3 minutes, and the supernatant was collected and stored in a new tube at -20°C for further use.

Method 5 (Phenol-chloroform with modified lysis buffer method)

DNA from semen samples was extracted using Phenol-chloroform method as described by Hanson and Ballantyne (2004) along with slight modifications. Before proceeding to phenol- chloroform extraction method, following treatment was given -two hundred microliter semen aliquots were centrifuged at 6000 rpm for 5 min. Each pellet was resuspended in 1 ml TES solution [100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)] and centrifuged again. The pellet, 500 µl lysis buffer [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS)] was added, along with 22 µl 0.1 M dithiothreitol (DTT) and 25 µl proteinase K. The mixture was incubated at 55°C for 3 h, with hourly vortexing. After this, 500 µL phenol, (equilibrated with Tris, pH 7.8), was added, followed by vortexing and centrifugation at 10,000 rpm for 3 min.

The supernatant was transferred to another tube, along with 300 µl phenol and 300 µl chloroform, followed by vortexing and centrifugation at 10,000 rpm for 3 minutes. The supernatant was transferred to a new tube, and then 700 µl chloroform was added. The mixture was vortexed and centrifuged again, and the supernatant was transferred to another tube. Two volumes of cold 95% ethanol were added, and the tube was incubated at -20°C for 4 hours. Each sample was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was subsequently removed. Each DNA pellet was dried, resuspended in 50 µl 1X TE buffer (100 mM Tris-HCl, pH 7.5, 0.25 M EDTA), and stored at -20°C for further use.

Spectrophotometer Measurements

The genomic DNA concentration and purity was assessed by spectrophotometer (BIO-RAD, India) by measuring the wavelength at A_{260} and A_{280} and their purity was assessed by taking the 260/280 ratio (Sambrook and Russell, 2001). The concentration of DNA was calculated using the following formula. DNA concentration (µg DNA/ml) = OD 260 x 50 x dilution factor.

Real Time PCR amplification

A real-time PCR targeting the housekeeping gene (GAPDH) was used to assess the presence of amplifiable DNA in extracts of semen samples.

Amplification was performed in Thermal cycler (Step One Plus, Applied Biosystem). The PCR reaction mixture comprised of 12.5 µl, 2X SYBR Green mastermix 4 µl (1pm) forward primer, 4 µl (1 pm) reverse primer 4 µl of template and 0.5 µl of nuclease free water, making the final volume of 25 µl and were run in triplicate along with non template control (NTC). Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmers by optimized protocol (Table 1). Ct values were used to assess the amount of amplifiable DNA in the samples.

Cycling conditions for qPCR

Table 1a: Thermocycling condition

Stage	Repetition	Temperature	Time
1	1 cycle	50 ^{0}C	2 minutes
2	1 cycle	95 ^{0}C	10 minutes
3	40 cycle	95 ^{0}C	15 seconds
4	40 cycle	58 ^{0}C	1 minute

Table 1b: Denaturing condition

Stage	Repetition	Temperature	Time
1	1 cycle	95 ^{0}C	15 seconds
		60 ^{0}C	20 seconds
		95 ^{0}C	15 seconds
		60 ^{0}C	15 seconds

Gel Electrophoresis

Integrity of the extracted DNA was assessed by running the DNA samples on 0.8% agarose gel containing ethidium bromide.

Statistical Analysis

The five different DNA extraction methods were assessed with the model:

$Y_{ij} = \mu + p_i + s_j + e_{ij}$, where

Y_{ij} = DNA score by evaluation criterion for the i^{th} extraction method and j^{th} sample

μ= overall mean, p_i = effect of i^{th} DNA extraction method (i = 1–5) s_j = effect of the j^{th} sample (j = 1–5)

e_{ij} = random residual

The DNA concentration, purity and Ct values (real time PCR targeting the GAPDH gene) were statistically evaluated by analysis of variance (ANOVA), and differences were compared with the *post hoc* Tukey test at a significance level of 0.05. The results are reported as means ± SE.

RESULTS AND DISCUSSION

Method comparison

The quantity, purity and GAPDH Ct values of DNA obtained by the five extraction methods are shown in Table 2.

Table 2: Statistical comparison of five genomic DNA extraction methods

Method	OD260/280	DNA concentration(ng/µl)	GAPDH Ct values
Qiagen DNeasy Blood & Tissue Kit	1.406(0.111) ^{a}	48.196(18.910) ^{a}	25.94(0.268) ^{a}
Purelink Invitrogen Kit Modified Qiagen	0.900(0.111) ^{b}	2.804(18.910) ^{a}	32.92(0.268) ^{b}
DNeasy Blood& Tissue Kit Chelex-100	1.680(0.111) ^{ac}	72.784(18.910) ^{a}	25.30(0.268) ^{a}
Phenol Chloroform with	1.136(0.111) ^{ab}	256.320(18.910) ^{b}	22.06(0.268) ^{c}
Modified Lysis Buffer	2.058(0.111) ^{c}	191.840(18.910) ^{b}	22.88(0.268) ^{c}

{Results are marginal means with standard errors in parentheses. a,b,c,Comparison of values within each column; values with the same superscript are not statistically different (P<0.05) from each other but they differ significantly (P<0.05) from values with different superscript}

DNA purity

The 260/280 spectrophotometric measurement was used for the evaluation of DNA purity. Values above or below 1.8 imply impurities in the DNA (Birren *et al.*, 1997). In the present study, we found that- out of five methods, modified Qiagen DNeasy Blood and Tissue Kit, extracted DNA of highest purity i.e. 1.68, which is very near to ideal value of 1.8(Table 2). This may be due to treatment with extra buffer containing 10 mM EDTA which is used to chelate the metals. Sequestration of Magnesium ions by EDTA serves to inhibit nuclease activity. Additional treatment of semen with proteinase K completely degraded cellular proteins. Therefore, this method provided good quality of DNA. Purelink Invitrogen kit extracted DNA of least purity i.e., 0.9, which is lower than the ideal value of 1.8. A low 260/280 nm ratio is indicative of contamination with proteins, which could inhibit downstream applications and also hamper DNA-banking. DNA extraction residues like phenol, guanidine, salt or solvents are also considered inhibitors for downstream applications. The DNA purity values for the Qiagen DNeasy Blood and Tissue Kit and Chelex -100 methods were 1.406 and 1.136 respectively. The purity of DNA from Phenol Chloroform Modified Lysis Buffer method was greater than the ideal value, 1.8 indicating high residual RNA so these methods should include treatment with RNase A, so as to obtain pure DNA prepartions. The findings are in concordance with that of Manuja *et al.*, 2010, where, Chelex-100 and Qiagen modified methods for extraction of DNA from semen were found to be superior qualitatively. Silva *et al.* (2014), in their study to evaluate three methods to extract DNA from goat sperm also found that DNeasy Blood & Tissue Kit produced a higher ($P < 0.05$) purity product than the Chelex-100 method and Phenol Chloroform method.

DNA quantity

The spectrometric assay demonstrated that the quantity of DNA extracted from sperm samples was higher ($P < 0.05$) for the Chelex-100 protocol and Phenol Chloroform Modified Lysis Buffer method than for the Qiagen DNeasy Blood& Tissue Kit, Purelink Invitrogen Kit or modified Qiagen DNeasy Blood and tissue kit methods (Table- 2). Silva *et al.* (2014) reported similar results for Chelex-100 protocol and Phenol Chloroform Modified Lysis Buffer method. Purelink Invitrogen kit method could not extract DNA from semen. Since, real-time PCR is a reliable tool for assessing DNA quantity and quality for downstream applications, a further attempt was made for use of DNA extracted by various methods for the assessment of PCR inhibition. Therefore, we tested the DNA extracted with the 5 methods for the presence of amplifiable DNA and PCR-inhibitors by using real-time PCR targeting GAPDH gene (Fig.1).

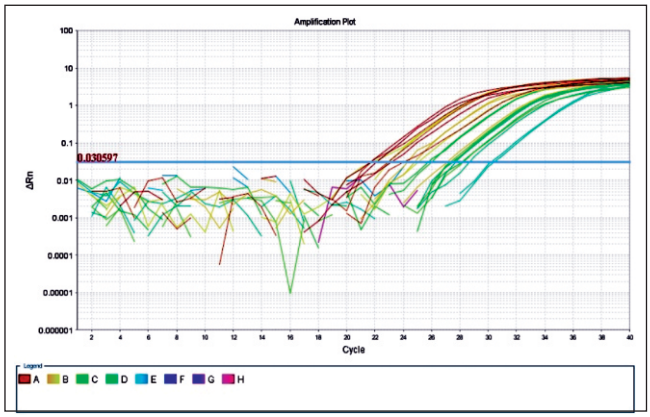

Fig. 1: Real time PCR amplification plot of the GAPDH gene from the DNA extracted from semen samples through different extraction methods and the control. No amplification was detected in the non template control

Chelex-100 protocol and Phenol Chloroform with Modified Lysis Buffer methods gave the lowest mean Ct values, followed by Qiagen DNeasy Blood & Tissue Kit and modified Qiagen DNeasy Blood & Tissue Kit method ($P < 0.05$). High Ct values of 32 were observed in Purelink Invitrogen Kit method. Lower Ct values are desirable since they are associated with larger amounts of amplifiable DNA. Ct values were found inversely proportional to the spectrometric values as shown in Table 2. Analysis of melting curve illustrated, there is neither primer dimer nor nonspecific products in reactions (Fig. 2). None of the NTC yielded any signal prior to 30 cycles, which is the upper limit for conventional PCR. Except Chelex-100 method and Phenol-Chloroform protocol (three samples from each method), none other DNA extracts caused a detectable inhibition rendering them, unsuitable for large-scale downstream applications despite their high spectrophotometric DNA values. The reason may be attributed to the single step execution of Chelex-100 method, which does not remove DNA inhibitors that could interfere with DNA typing and so in their presence, a passage in centricon or microcon could be required to purify the sample

(Sepp *et al.*, 1994). PCR inhibition in the DNA extracts of Phenol chloroform modified method may be attributed to the DNA extraction residues like phenol, salt or solvents, which are considered as inhibitors for downstream applications. In addition to this, Phenol- chloroform procedure employs many dangerous reagents. It is time consuming since it requires many steps and a particular accuracy to avoid the loss of materials (Goldenberger *et al.*, 1995). Unlike our findings, Chelex- 100 method appeared as potential tool for extracting sperm DNA without limitations for PCR (Manuja *et al.*, 2010; Silva *et al.*, 2014).

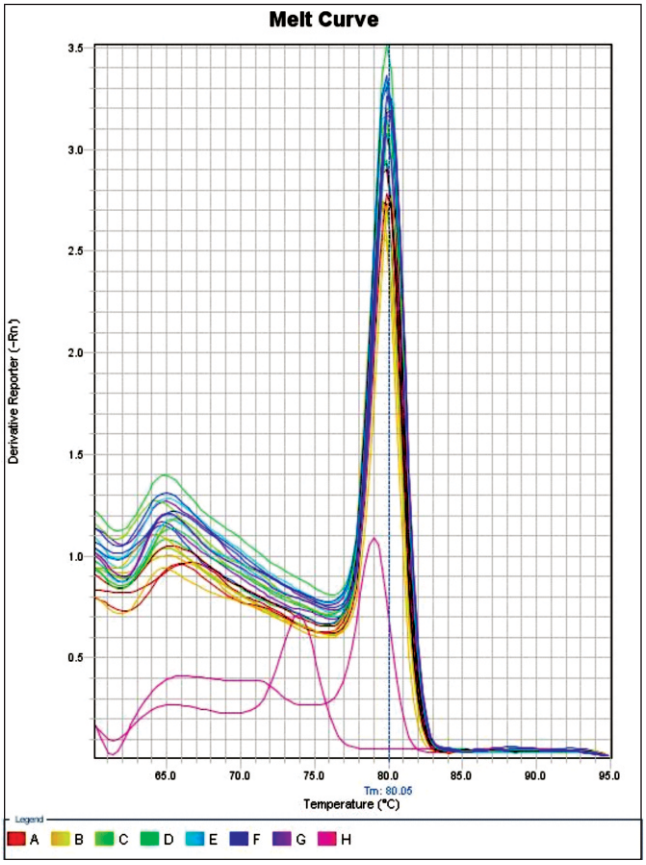

Fig. 2: Melt curve of the GAPDH gene from the DNA extracted from semen samples through different extraction methods and the control

DNA integrity

Assessment of Integrity of the extracted DNA was assessed by 0.8% agarose gel electrophoresis (Fig. 3). Gel electrophoresis

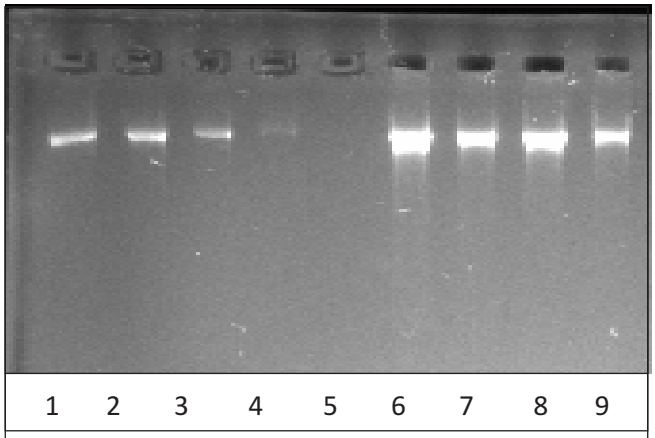

Fig.3: Electrophoresis results on 0.8% agarose gel with DNA extracted from semen samples by five different DNA extraction methods. Lanes 1 and 2 represents DNA extraction from modified Qiagen DNeasy blood and tissue kit method, Lanes 3 and 4 represents DNA extraction from Qiagen DNeasy blood and tissue kit method, Lane 5 represents DNA extraction from Purelink Invitrogen kit method, Lanes 6 and 7 represents DNA extraction from Chelex method and Lanes 8 and 9 represents DNA extraction from Phenol chloroform with modified lysis buffer method.

revealed that high-molecular-weight non-degraded genomic DNA was obtained with all methods except Invitrogen Purelink Invitrogen kit method. The intensity of bands corresponded to DNA concentration. High intensity bands were observed for Chelex-100 method followed by Phenol Chloroform modified lysis buffer method, modified Qiagen DNeasy Blood & Tissue Kit method and Qiagen DNeasy Blood & Tissue Kit method.

CONCLUSION

Based on the overall evaluation of methods and limitations associated with them, modified Qiagen DNeasy Blood & Tissue Kit method was found superior to all other methods. DNA extraction by modified Qiagen DNeasy Blood & Tissue Kit method resulted in satisfactory values of concentration and purity. Moreover, no PCR inhibition was observed with any of the replicates. Integrity of DNA was also found intact.

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