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Comparative Evaluation of DNA Isolation Methods from Porcine Semen

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ABSTRACT

Different biological samples require specific protocols for isolating DNA from them. Semen samples require special considerations because of the presence of protamines in place of histones in the DNA of spermatozoa. An experiment was conducted at the Institute Swine Production farm at ICAR-Indian Veterinary Research Institute, Bareilly, to select the best-suited DNA isolation protocol from porcine semen. Healthy Landrace boars from the farm were given preliminary training and 18 ejaculates were collected from them for the study. DNA isolation was done in three methods in order to standardize and finalize a protocol suitable for porcine semen. The methods were phenol-chloroform as described in Russel and Sambrook (2001) with slight modifications, Chelex-100 (Walsh *et al*., 2013) and using commercial kit method (Applied Biosystems). The genomic DNA isolated from the porcine semen samples were checked for quality, purity, and concentration. Among the three methods selected for DNA extraction, all gave apparently good quality DNA with purity in the preferred range of 1.8-2.0 but differed in the concentration according to the nanodrop reading. On analysing the results statistically, the modified Phenol-Choloroform technique showed significant variation in concentration of isolated DNA in comparison to the Chelex -100 method and commercial kit method.

HIGHLIGHTS

- By employing various isolation procedures, the yield and quality of DNA recovered from semen samples vary.
- For isolating DNA from swine semen, a small modification to the Phenol-Chloroform procedure was shown to produce statistically more effective results.

Keywords: DNA isolation, Boar semen, Phenol chloroform method, Chelex method, Commercial DNA extraction Kit

DNA isolation is now considered a routine protocol for biological studies including molecular identification, phylogenies, and genomics studies. The success of downstream processing like PCR, Realtime PCR, etc depends on the extraction and availability of pure and sufficient amounts of DNA. Different methods became popular over the last few decades for extracting DNA from several biological materials. Each technique will vary according to the biological sample and even the species from which it is taken. Therefore, standardization of protocol is required for each type of biological sample. DNA isolation from semen samples requires special considerations. The seminal plasma which contains fructose and many proteins may reduce the purity of genomic DNA isolated from semen samples (Weyrich *et al*., 2012). Also, during spermatogenesis, more than 90 % of histones are replaced with protamines. This makes the sperm chromatin structure very dense and this protects the sperm from external stresses (Donkin and Barrès, 2018). So, unlike the somatic cells, due to the disulfide bridges formed within and between the protamines, the extraction

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of sperm DNA through standard techniques may not yield good results.

The main steps involved in the isolation of good quality nucleic acid from the sample include cell/tissue disruption, separation of the nucleic acid from other cell contents, and its final purification at desired concentration (Dairawan and Shetty, 2020). When optimizing the DNA isolation protocol for an organism focus should be on the yield and quality of DNA while keeping the process economical, quick and less extensive to execute in the laboratory (Chacon and Griffiths, 2014). Even though the commercial kits are rapid and simple for carrying out the extraction process, the high cost limits its routine use in the laboratory. Also, the yield of DNA is sometimes compromised. Phenol is usually used for removing proteins from cell lysate during DNA isolation protocol. Despite the handling difficulty and corrosive nature of phenol, it does not completely assure the absence of RNAase. When a mix of Phenol, Chloroform, and isoamyl alcohol is used, this problem was rectified (Sambrook and Russel, 2001). When the water (+DNA +protein) and phenol are mixed in the protocol, the DNA does not dissolve in the phenol but remains in the water phase. Following the phase separation by centrifugation the DNA containing the upper layer can be transferred to a new tube and DNA can be precipitated using chilled ethanol (Ghaheri *et al*., 2016). Griffin and co-workers successfully used guanidine thiocyanate to isolate high quality genomic DNA from sperm cells (Griffin, 2013). Researchers added further components to the protocol such as proteinase-K for the more efficient digestion of the nucleoproteins. DTT (dithiothreitol) is also used as a reducing agent, which cleaves disulfide bonds and allows proteins to unfold (Fjelstrup *et al*., 2017). Some other protocols use resins like Chelex-100. It is composed of styrene-divinylbenzene copolymers containing paired iminodiacetate ions. It removes the Mg^{2+} from the Nuclease reactions, thus inactivating them to protect the Nucleic acid. This was reported to be the quick and easy method for isolating DNA from bull semen for PCR to detect BHV-1 virus (Manuja *et al*., 2010).

For studying the sperm genetics and epigenetics of different species, there is a need to start with a sufficient amount of pure genetic material. In the present study, we compare the Phenol-Chloroform method, the Chelex method, and the commercial kit method to find the best-suited protocol for isolating good-quality DNA with maximum purity and integrity from porcine semen.

MATERIALS AND METHODS

The experiment was conducted at the Institute Swine Production farm at ICAR-Indian Veterinary Research Institute with the experimental procedures approved by Institute Animal Ethics Committee. The ejaculate was filtered with cheesecloth to prevent the gelatine plug from inhibiting further processing. They were individually moved to the collection pen (Dimension 10'×10') with a non-slick floor with rubber bedding and artificial/ dummy sows. A boar was given preliminary training first using a female (close to size-matched) in standing heat and the same was conveniently replaced with the dummy. The representative semen samples were collected from the sperm-rich portion out of 4 fractions (gelatinous pre-sperm, sperm-rich, sperm-poor, and gelatinous plug) of boar semen in sterile 15 ml falcon polypropylene tubes. The ejaculate was filtered with cheesecloth to prevent the gelatine plug from inhibiting further processing.

DNA isolation methods

DNA isolation was done in three methods in order to standardise and finalize a protocol suitable for porcine semen. The methods were phenol-chloroform as described in Russel and Sambrook (2001) with slight modifications, Chelex-100 (Walsh *et al*., 2013) and commercial kit method (Applied Biosystems).

Method 1

Phenol-chloroform method

The genomic DNA from semen was isolated as per Sambrook and Russel (2001) with slight modifications to fit our laboratory condition and sample quantity. The freshly collected semen samples were used for DNA extraction. 1 ml fresh semen sample was centrifuged @ 3000 rpm for 20 min at room temperature. The cell pellet was carefully taken out and washed with PBS and mixed thoroughly. This washing step was repeated 2 more times and the remaining pellet was processed. Sperm buffer (1 M Tris buffer, 3M NaCl, 0.5M EDTA) was added 1 ml and vortexed to gently disperse the pellet in the extraction buffer and was incubated at 37˚C for 60 min. Subsequently, 100

µl of 10% SDS was added and mixed gently by inverting the tube once or twice. Care was taken while mixing because after adding SDS, lysis of the cell wall occurs and DNA was fully exposed. As a result, the content of the tube becomes viscous. 5 µl Triton and 20 µl proteinase K were added to this. The proteinase K was added twice @ 20µl giving an interval of 2 hr. All reagents were mixed and incubated overnight at 56°C. The genomic DNA was extracted using equilibrated Phenol followed by Phenol: Chloroform; Isoamyl alcohol (25:24:1) and precipitated by chilled Iso-propanol. The DNA pellet was washed with 70 % Ethanol. Only the DNA samples with good quality, purity, and concentration (checked by Nanodrop 1000 spectrophotometer) were used for further analysis.

Method 2

Chelex method

The DNA extraction was carried out as per the method described in the OIE manual (2010) as described by Walsh *et al*. (2018) with slight modifications to meet our laboratory conditions. Chelex 100 sodium (10% w/v in sterile milli Q water), 100µl was added to 15µl semen sample followed by 7.5µl Proteinase -K (20mg/ml) and 7.5µl DDT (1M). Finally, Nuclease Free water, 90µl was added and mixed using a magnetic stirrer. The tubes were incubated at 56° water bath and boiling water bath for 2hrs and 8 minutes respectively. After centrifugation at 8000 rpm for 4 min supernatant was taken for phenol chloroform isoamyl alcohol extraction. The supernatant was again added to a new centrifuge tube to which an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm at 4°C. This step was repeated and the upper phase after centrifugation was transferred to a new tube and the chilled ethanol and 3M sodium citrate were used for the precipitation of DNA. The pellet was washed with 70% ethanol, the supernatant was discarded. The pellet was dried at room temperature for 15 minutes and dissolved in Nuclease-free water for further use.

Method 3

Commercial kit method

Genomic DNA was also extracted using Applied Biosystems™ DNA Extract All Reagents Kit (Fisher Scientific, United States) following the instructions provided in the manual.

Purity, quality, and quantity check

The genomic DNA isolated from the porcine semen samples was checked for quality, purity, and concentration. The samples with good quality, pure and concentrated were used for further analysis. The quality of extracted genomic DNA was assessed through horizontal submarine agarose gel electrophoresis. %µl of the extracted DNA was loaded to wells created on 1% agarose. Gel was visualized and photographed under the Syngene Gel Documentation system. Intact DNA samples devoid of smearing were inferred of good quality and were used in the present investigation. For evaluating purity, absorbance was taken at 260 nm and 280 nm. Highly pure DNA was having 260/280 ratio of 1.8-2.0. The absorbance ratio was taken using a Nanodrop 1000 spectrophotometer.

STATISTICAL ANALYSIS

The mean values were compared using Duncan test at ($p < 0.05$) with the help of SAS software.

RESULTS AND DISCUSSION

Many DNA isolation protocols has been practiced till date which uses different reagent for cell lysis and selective precipitation of DNA from specific biological materials. A comparative evaluation of different procedures was necessary in order to select the best suited method for DNA extraction from porcine semen.

DNA was extracted from all the samples using three methods separately and was dissolved in TE buffer. The horizontal submarine agarose gel electrophoresis showed intact DNA on visualization. Purity and quality analysis by nanodrop 1000 spectrophotometer was done. The range of purity and concentration of DNA extracted is given in table 1. Graph showing purity and concentration of DNA extracted by each method is shown in the Fig. 1 and 2 respectively.

Table 1: Range of purity (260/280) and concentration (ng/µl) of DNA extracted by each technique

Method used for extraction	Range of purity of DNA extracted (260/280) ratio	Range of concentration of DNA extracted (ng/µl)
Chelax method	1.8-1.92	117.8-206
Phenol-Chloroform method	1.7-1.9	225-1120
Kit method	1.7-1.92	19.4-40

The ratio of absorbance of 260 nm and 280 nm are used to assess the purity of DNA. A ratio of ~1.8 is accepted as "pure" for DNA. If the ratio is lower it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm.

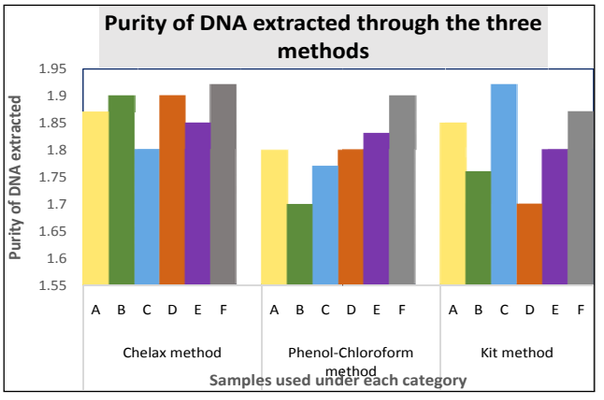

Fig. 1: Graph showing purity (260/280 ratio) of DNA extracted by three methods

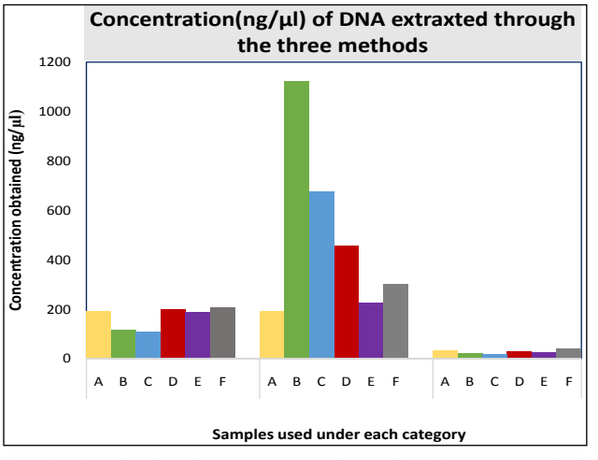

Fig. 2: Graph showing concentration of DNA extracted (in nanogram/µl) by three Methods

Average and SE of Nanodrop reading (Purity and concentration) of DNA isolated by three methods are shown in the table 2. Genomic DNA from each animal was diluted to a working solution using TE buffer to get a final concentration of 50 ng/µl. This genomic DNA was used as template DNA.

Table 2: Average and SE of Nanodrop reading (Purity and concentration) of DNA isolated by three methods. On comparing the three methods, Phenol-Chloroform method was showing significant variation from other methods in concentration of DNA extracted with a P-value less than 0.001

Parameter	Chelex method	Phenol-Chloroform method	Kit method
Purity (260/280)	1.87±0.017	1.8±0.027	1.81±0.032
Concentration (ng/µl)	168^{A}±17.72	495^{B}±144	28^{A}±3.19

*Values with same superscript in a row do not differ significantly.

The range of purity of the Chelex method was 1.8-1.92, while the phenol-chloroform and kit method showed an almost similar range of 1.7-1.9 and 1.7-1.92 respectively. Despite the minute range difference, the three methods do not differ much statistically. This suggests that all three methods were able to isolate DNA from porcine semen samples with an acceptable range of purity (without much protein contamination) needed for downstream processing. Similar purity of isolated DNA was reported when the phenol-chloroform method was used in human sperm (Yuan *et al*., 2015). Chelex method even though is effective for pure DNA isolation from pig spermatozoa through our study, the purity results are found to be variable in different species. While this method was able to give similar results in buffalo semen (Manuja *et al*., 2010), it produced DNA purity below 1.8 in goat semen (Silva *et al*., 2014). The variations can also be due to changes in sample acidity, wavelength accuracy of the spectrophotometer, and the ratio of nucleotide mix in the sample.

The qualitative evaluation of the DNA extracted by the three methods showed clear-cut differences. The average concentration with the Chelex method was 168±17.72 ng/µl, while extraction with kit generated only 28±3 ng/µl, and the Phenol-chloroform method yielded a higher average concentration of 495±144 ng/µl. The quantity

of DNA yielded from the Phenol-Chloroform method was quantitatively different as per Duncan's multiple range test for the variable. This was in contrast with the isolation from goat semen (Silva *et al*., 2014) where the Chelex method yielded more DNA (346±105 ng/µl) than the Phenol-Chloroform method (153±24 ng/µl). The better yield from Phenol-Chloroform method can be attributed to double digestion using Proteinase-K. The lesser yield from the kit method is suggested to be improved by increasing the exposure time of proteinase K.

CONCLUSION

To conclude, the highest mean DNA concentration value of the Phenol- Chloroform method suggests it to be more suitable for isolating pure DNA from porcine semen when a reasonable amount of DNA is needed for the proceeding steps. The Chelex method even though yielded less DNA concentration in comparison to the Phenol-Chloroform method, the yield is sufficient for further downstream processing. Since it is less time-consuming and needs a lesser number of steps and reagents than the Phenol-Chloroform method, it can be selected for fast and safe DNA isolation yielding a sufficient amount of pure DNA.

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