EVALUATION OF DIFFERENT METHODS OF DNA EXTRACTION FROM SEMEN OF BUFFALO (Bubalus bubalis) BULLS

Anju Manuja1, Sonia Manchanda2, Balvinder Kumar1, S. Khanna2 and R.K. Sethi2

ABSTRACT

Microbes excreted in the semen of infected or carrier bulls can be disseminated to susceptible animals through artificial insemination. The polymerase chain reaction (PCR) has been employed successfully to detect infectious agents in tissues and body fluids. PCR inhibitors present in the semen pose serious problems in detection of microorganisms by inhibiting the amplification of the target DNA template. These inhibitors need to be removed completely during DNA extraction to amplify the target sequences in semen by PCR. DNA was extracted using seven different protocols from semen of buffalo bulls, and the quantity and quality was evaluated spectrophotometrically. Chelex-100 and Qiagen modified methods for extraction of DNA from semen were found to be superior qualitatively as compared to the other methods. In the Qiagen modified protocol, the semen was treated with two extra buffers containing EDTA to chelate the metals. Additional treatment of semen with proteinase K was included to completely degrade cellular proteins. DNA extracted by the phenol-chloroform and CTAB methods yielded high value of residual RNA and other contaminants. The chelex-100 method has the potential advantage of requiring a smaller volume of semen to extract good quality of DNA.

Keywords: DNA extraction, PCR inhibitors, semen, Bubalus bubalis, buffalo bull

INTRODUCTION

Several pathogenic microbes are excreted in the semen of infected/carrier bulls and due to lack of effective quality control practices, the infections are disseminated to susceptible animals through artificial insemination. It is, therefore, important that measures be undertaken to screen the bulls against various semen borne infections prior to their entry into breeding programmes. The polymerase chain reaction (PCR) technique has become an important tool to detect infectious agents. During DNA extraction procedures, when the cells are lysed to release nucleic acids, endogenous nucleases, enzymes that degrade nucleic acids (DNA and RNA), are also released. Thus, when minimally degraded (i.e., high integrity) nucleic acid is desired, nuclease activity should be minimized as much as possible. The success of PCR depends on the quality of the DNA; it must be free of contaminants and nucleases of the DNA that impair the amplification process. Inhibitory components of PCR may be present naturally in the sample or added during processing of clinical samples while preparing for PCR.

Spermatozoa and non-sperm cells also inhibit PCR. Semen is composed of cellular (spermatozoa, urogenital epithelial cells, round cells etc.) and non-cellular components. There is also a portion of the cells loaded with a special protein that anchors the sperm cell to the egg. Biochemically, there is a lot of nucleic acid (DNA), some glycogen...
as an energy source, lots of protein, but few fats and lipids. Besides this, the prostate produces zinc which protects the sperm chromatin condensation from becoming superstabilized as a result of excessive disulfide bond crosslinking of the protamines. So, it contains metal ions too. Thus, there is a need for methods and compositions for isolating high integrity, i.e., high molecular weight, nucleic acid molecules in a rapid and efficient manner. It is also very critical to keep an account of the inhibitory factors while interpreting the results to avoid false negative results. Although modified DNA extraction procedures from semen have been reported in cattle, the information on buffalo bull semen is scanty. Previously we reported a rapid, reliable and convenient method for DNA extraction from buffalo bull semen using Chelex 100 resins, and DNA was used successfully for the molecular detection of BHV-1 in *Bubalus bubalis* (Manuja et al., 2006). But no previous work seems to have been done on systematic comparison of these extraction procedures with conventional methods. In this study, as a preliminary for further work, we have evaluated different methods for DNA extraction from semen of *Bubalus bubalis* quantitatively and qualitatively.

**MATERIALS AND METHODS**

**Semen samples**

Fresh semen samples of four healthy Murrah buffalo bulls from organized farms in India were collected in Eppendorf tubes and stored at -20°C till further use. Bulls passed a complete physical examination and a breeding soundness evaluation. Strict hygienic conditions were maintained during semen collection.

**DNA extraction from semen**

DNA was extracted from semen of four bulls by the following extraction methods.

**Phenol: chloroform method**

DNA was extracted as per method described by Birren et al. (1997) with slight modifications. Briefly, 200 μl of semen was treated with 0.1 volume of 10% sodium dodecyl sulphate (SDS) and 0.1 volume of 2M sodium acetate and incubated for 1 h at 56°C in a water bath. After the addition of an equal volume of saturated phenol:chloroform:isoamylalcohol (PCI mixture in the ratio of 25:24:1) and vortexing and centrifuging at 10,000 x g for 10 minutes in refrigerated centrifuge, the upper aqueous layer taken in another tube was extracted with an equal volume of 24:1 chloroform: isoamylalcohol (CI) mixture. The PCI extraction was repeated until the interface was clear. To the final aqueous solution, a 0.1 volume of 3M sodium acetate pH 5.2 and an equal volume of isopropanol was added and mixed gently. The DNA was precipitated by keeping at -20°C overnight. The DNA was pelleted by centrifuging at 10,000 x g for 10 minutes and washed with pre-chilled 70% ethanol to remove the excess salts. The resultant pellet was air dried and dissolved in tris-EDTA buffer (TE).

**Cetyl Trimethyl Ammonium Bromide (CTAB) method**

DNA was extracted from semen as described by Ausubel et al. (1990) with some modifications. A mixture of 500 μl semen and 10 ml extraction buffer was incubated at 60°C for 1 h. After addition of 20 μl CI and centrifugation at 3000 x g for 20 minutes, the upper phase was taken in fresh tube and was precipitated with 1 volume of isopropanol at -20°C. The pellet obtained after centrifugation at 3000 x g for 20 minutes was extracted again with CI. The upper phase was mixed with 0.4 ml of 10% CTAB (Amresco, USA), 1 volume of CI and centrifuged at 3000 x g for 20 minutes. Then the upper phase mixed with equal volume of 1% CTAB was incubated at room temperature for 30 minutes. The pellet obtained after
Centrifugation at 3000 x g for 20 minutes was washed with 70% ethanol, 10 mM ammonium acetate and finally redissolved in 1 ml of TE.

**Chelex-100 method**

DNA was extracted by Chelex-100 method as previously described by Walsh et al. (1991) and modified by Santrude et al. (1996) and Manuja et al. (2006). Briefly, a 25 μl of sample was added to 200 ml of 5% Chelex-100 resin (Sodium form, Sigma, USA). Proteinase K (MBI fermentas, EU) at 0.1mg/ml concentration and 31 mM dithiothreitol (DTT) were added to 225 ml of semen and mixed thoroughly. After incubation at 56°C for 45 minutes, the mixture was incubated in a boiling water bath. After vigorous vortexing for 10 seconds, the material was centrifuged at 10,000 x g for 3 minutes. Then the supernatant was collected and stored at -20°C to be used for PCR assay as template.

**Qiagen Protocol**

DNA was extracted from 200 μl semen using mini QIAamp DNA mini kit (Qiagen, Germany) as per manufacturer’s instructions.

**Qiagen Modified 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 100 μl of proteinase K was added. After incubation at 56°C for 2 h, another 20 μl proteinase K was added and incubated again as specified above. After addition of lysis buffer supplied with the kit and ethanol in 400 μl quantity each, the mixture was applied to the mini spin column (Qiagen, Germany) and centrifuged at 6000 x g for 1 minute and the tube containing filtrate was discarded. The bound DNA was washed and eluted as per the manufacturer’s instructions.

**Trizol method**

TRI reagent method was used for total DNA isolation as per the manufacturer’s instructions (Sigma, USA). To 200 μl of semen an equal volume of TRI reagent was added and allowed to stand for 5 minutes at room temperature. Following addition of 40 μl chloroform, it was centrifuged after 15 minutes at 12,000 x g for 15 minutes at 4°C. After transferring the interface to a fresh tube, 100 μl of ethanol was added and centrifuged after 10 minutes at 12,000 x g for 10 minutes at 4°C. The pellet was washed with pre-chilled 75% ethanol, air-dried and dissolved in TE.

**One step-RNA reagent method**

DNA was extracted using one-step RNA reagent (Bio Basic Inc.) as per the manufacturer’s protocol. To 500 μl of semen, after centrifugation at 3000 rpm for 1 minute an equal quantity of reagent was added and incubated for 5 minutes at room temperature. After addition of 100 μl of chloroform, tubes were shaken vigorously, incubated at room temperature for 3 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was discarded. DNA was precipitated from the interface and organic phase with 150 μl of absolute ethanol and incubated at room temperature for 3 minutes and then centrifuged at 12,000 x g for 5 minutes at 4°C. The DNA pellet was washed twice with 0.1 M sodium citrate in 10% ethanol. At each wash, DNA pellet was kept in washing solution for 30 minutes at room temperature, centrifuged at 2000 x g for 5 minutes at 4°C. Finally the DNA pellet was washed with pre-chilled 75% ethanol, air-dried and dissolved in TE.
Quantification and Purity of DNA

The quantity and quality of extracted DNA was determined by spectrophotometer (Bechman, USA). The concentration of DNA was calculated based on the approximation that an absorbance reading of 1 of the purified DNA at 260 nm was taken to correspond to 50 μg/ml (50 ng/μl). DNA purity was estimated by determining the A260/A280 ratio and the reference value considered for purity was 1.8.

Statistical Analysis

Quantitative and qualitative analysis was done by Duncan’s multiple range test (Steel and Torrie, 1984).

RESULTS AND DISCUSSION

Current methods for isolating nucleic acids from cells generally include lysing the cells and inactivating nucleases using chaotropic salts and a nonionic surfactant. The released nucleic acids are then selectively precipitated from the solution. The combination of chaotropic salts and nonionic surfactants, however, are ineffective when they have to go through tough contents to release nucleic acid. Semen contains a lot of nucleic acid (DNA), glycogen, proteins, fats and lipids and some metal ions, some of which may inhibit PCR. Different scientists have developed different methods of DNA extraction for the removal of these unknown inhibitors from the semen so as to obtain better results in PCR (Santrude et al., 1996; Masri et al., 1997; Manterola et al., 2003; Gupta et al., 2006; Manuja et al., 2006). But a comparative study of these procedures with the conventional methods is lacking. Therefore, in this study, we have evaluated seven different protocols for extraction of DNA from the semen of buffalo bulls.

Qualitative analysis of all the four replicates of DNA samples extracted from semen of buffalo bulls by different extraction methods is shown in Table 1. Relative qualitative analysis of different DNA extraction methods employing all the four replicates was calculated by subtracting the A260/280 of DNA samples from the value of 1.8, which was the purity value of DNA taken as the reference (Figure 1). Values above or below 1.8 imply impurities in the DNA (Birren et al., 1997). All the four samples extracted by the phenol-chloroform method yielded values higher than 1.8 whereas two samples from CTAB exhibited values less than 1.8 (Table 1). Phenol-chloroform and CTAB methods have mean values much greater than 1.8 indicating high residual RNA so these methods should include treatment with RNase A, so as to obtain pure DNA preparations. These two methods are similar as per Duncan’s multiple range tests for variables. These are followed by Qiagen modified, Chelex-100, Qiagen, one-step RNA reagent, Trizol, and all these methods are similar (Table 1, Figure 1). The Qiagen and one-step RNA reagent methods have less than 1.8 A260/A280 ratios for extracted DNA which indicates protein contamination, maybe due to decreased protease activity. The Chelex-100 method has a mean value of 1.8, so the DNA obtained is pure enough. The Qiagen modified method is also a method of choice as the DNA obtained was purer than other methods in this case. A potential advantage of the Chelex-100 method is the volume of semen, i.e. 25 μl; only one straw can be used for extraction of DNA from semen (Manuja et al., 2006). This method also removes the polyvalent metal ions, which have potential lesive effects on DNA extraction at high temperatures (Santrude et al., 1996; Manuja et al., 2006) so this method should be the method of choice. The Chelex-100 method has also been used for molecular detection of bovine herpes virus-1 (BHV-1) in Bubalus bubalis and was found to be rapid, convenient and accurate.
Table 1. Qualitative analysis of DNA extracted from semen by different methods.

<table>
<thead>
<tr>
<th>Semen samples</th>
<th>Quality of DNA extracted from semen in ng/µl</th>
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<tbody>
<tr>
<td></td>
<td>Phenol-Chloroform</td>
</tr>
<tr>
<td>1</td>
<td>2.87</td>
</tr>
<tr>
<td>2</td>
<td>2.86</td>
</tr>
<tr>
<td>3</td>
<td>2.63</td>
</tr>
<tr>
<td>4</td>
<td>2.64</td>
</tr>
<tr>
<td>Mean</td>
<td>2.75^A</td>
</tr>
<tr>
<td>Std. Dev</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Means bearing different superscripts (A&B) are significantly different with respect to Duncan’s multiple range test (P<0.01).

Figure 1. Extent of DNA contamination using different extraction procedures.
(Manuja et al., 2006). In the Qiagen modified protocol, the semen is treated with two extra buffers containing 10 mM EDTA which is used to chelate the metals. Sequestration of Mg ions by EDTA serves to inhibit nuclease activity. Additional treatment of semen with proteinase K is included to completely degrade cellular proteins. Therefore, this method provides good quality of DNA.

Quantitative analysis of different DNA extraction methods showed the highest mean value for all the four replicates of DNA extracted by Trizol followed by Qiagen, phenol-chloroform, one-step RNA reagent, Chelex-100, Qiagen modified, CTAB, which were all quantitatively different as per Duncan’s multiple range test for variable. The Quantity of DNA obtained was greater with the Trizol and Qiagen methods in comparison to the other methods studied (data not shown). Since the DNA was extracted from variable amounts of semen according to the given protocols, the comparison was determined on the basis of 100 μl of semen.

A further attempt to use of DNA extracted by various methods for the detection of infectious agent using PCR and subsequent comparative analysis is required. However, we have reported earlier a rapid, simple method of DNA extraction using Chelex-100 from semen of buffalo bulls for its use in PCR for detection of BHV-1. Inhibition in a PCR assay due to excess host genomic DNA is suggested to be a simple consequence of decrease in the rate of diffusion of macromolecular components in the reaction mixture (Cogswell et al., 1996). It has been reported that higher amounts of host genomic DNA could interfere with the PCR detection of the infectious agent (Cogswell et al., 1996). Higher dilution of DNA from clinical samples have also been reported to increase the sensitivity of the PCR assay (von Stedingk et al., 1995).

It is concluded that the Chelex-100 and Qiagen modified methods for extraction of DNA from semen were superior qualitatively as compared to the other methods. If cost is to be considered then phenol-chloroform method is the cheapest method to follow, but the expensive method used in preparing high quality DNA is well worth since this will be stable for years, eliminating the need to repeat extractions. Moreover, the samples derived from the unique conditions are frequently not replaceable.

REFERENCES


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