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ISOLATION OF GENOMIC DNA FROM FRESH AND OLD CLOTTED BLOOD SAMPLES OF INDIAN GHARIYAL (*Gavialis gangeticus*)

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ABSTRACT

The aim of the study was to find a method to overcome the problems encountered during DNA isolation from old clotted blood samples leftover after routine blood serum chemistry or clinical studies for monitoring and diagnosis of diseases. As such blood samples can be a good source of genomic DNA for molecular biology research especially in wild animals, where handling and restraining of animal is not a common feature. For the present experimental work, the blood samples were collected from Indian Ghariyals at National Chambal Gharial Wildlife Sanctuary, Madhya Pradesh, India, by puncturing their tail vein, 2ml of blood samples each were taken. Samples were brought to laboratory and were grouped as fresh blood samples (total number: 07), old clotted samples as stored at 4oC (total number: 11) and -21°C (total number: 09); respectively were analysed. We demonstrated in detail the protocol for extracting genomic DNA from clotted blood using basic equipments and chemical reagents commonly found in laboratories. Our resultant protocol efficiently yielded high DNA concentration (ng/µl) in fresh clot (2309.89±283.46); at 4°C (1495.37±119.76) and -21°C (684.31±137.35) quality in terms of intactness and purity of the DNA sample that can be further amplified efficiently using PCR. However, DNA concentration for fresh and stored samples at 4oC and -21°C showed significant (P<0.05) difference from processed fresh and stored samples. Thus, it can be concluded that the extraction of genomic DNA from fresh and stored sample (4°C and -21°C) could be efficiently done with by breaking the clot simply through basic equipments and chemical reagents used in the present protocol.

Key words : *Clotted blood, genomic DNA, ghariyal (gavialis gangeticus), temperature, quality and quantity.*

Genomic DNA used in molecular biology research is usually extracted and purified from uncoagulated blood; however the storage and transport of blood samples collected from field to the laboratory often leads to clotting of blood even after addition of anticoagulants, hence purification of intact nucleic acids can prove problematical. Classically genomic DNA from clotted blood is not preferable due to the difficulty of extraction techniques and requirement of special equipments. Whole blood serves as the best source for the isolation of genomic DNA in molecular biology research, either in humans or animals. However, keeping in view the nature of wild animal as well as their behavior, it is seldom impossible to collect blood for each and every time to test. Hence, it is necessary that whenever any amount of blood is collected it should be used most efficiently. Collection of blood from Crocodilians specially poses challenges among wild life workers. In such conditions, blood clots left after routine biochemical or hematological studies can be effectively used as a source of genomic DNA for diagnosis, molecular characterization or forensic purposes (1).

Ghariyal (*Gavialis gangeticus*) is a protected wild animal, protected populations are present in the banks of rivers traversing through National Chambal Gharial Wildlife Sanctuary (Chambal river, Madhya Pradesh), Katarniaghat Wildlife Sanctuary (Ghagra river, Uttar Pradesh), Son Gharial Sanctuary (Son river, Madhya Pradesh) and the rainforest biome of Satkosia Gorge Sanctuary (Mahanadi, Orissa). With the advent in science, there are various methods for isolation of genomic DNA from clotted blood (1, 2, 3), however most of these either require specialized equipments or buffers, we here describes one such protocol that is capable of isolating good quality and quantity of genomic DNA from fresh and old clotted blood samples.

This study describes a set of three types of samples used for extraction of intact genomic DNA. The DNA obtained by this procedure can be rapidly amplified by PCR for further analysis. PCR assays was done by using universal primers for 16s rRNA gene4.

MATERIALS AND METHODS

Sample collection : The blood samples (2ml each) were collected from Indian ghariyals at National Chambal Gharial Wildlife Sanctuary, Madhya Pradesh, India, by puncturing their tail vein. Total twenty seven samples of clotted blood were used in this study, stored at 4°C, -21°C and processed fresh (Table-1). Samples stored at 4°C and -21°C were the leftovers after haematological studies stored for 1 month or more.

DNA extraction : The basic procedure for DNA extraction remained the same for all the three categories of samples. The blood clots were first homogenized using 9gm/L NaCl solution (2), old clots are difficult to homogenize, therefore, first 500 µl of 9gm/L NaCl was added and then vaccutainers are kept on a stand for 5 min, then with a gentle shaking the intact blood clots were taken out by a forceps on a glass slide, the idea behind this is somehow to make the blood clot disintegrated, if not fully homogenized, so that the lysis buffer and other reagents can have better access to the white cells entangled in the clot, then with a sterile sharp BP blade the clot was chopped into smaller pieces, alternatively after removal from the vaccuatiners the clot can be rapidly freezed to solid form in a deep freezer or by putting them on ice and then quickly cut into small pieces. However, the clots stored at 4°C can be very well disintegrated by vortexing vertically for 15 – 20 min. After this, the tubes were centrifuged at 3000 rpm for 5-7 min, the supernatant is removed, the blood cells are then lysed with about 1 ml TE9 buffer (500 mM Tris pH 9.0; 20 mM EDTA; 10 mM NaCl), 0.5% Triton X 100, 20 µl of proteinase K (20 mg/ml) and 15-20 µl of 20% SDS. The tubes are then kept on vortex shaker for 2-5 min and kept in water bath at 55°C for 12 hr, the older clots stored at -21°C invariably required a second incubation by adding second aliquot of 20 µl proteinase K (20 mg/ml) and 20 µl of 20% SDS at 55°C for 6-8 hr. The tubes were than centrifuged at 12000 rpm for 10 min.

Table-1: Presents the concentration (ng/µl) and OD 260/280 values of clotted blood samples stored at different temperature.

Storing Temperature	Concentration (ng/µl)	OD 260/280
Fresh Clot	1950.00	1.80
Fresh Clot	2300.00	1.76
Fresh Clot	3900.00	1.70
Fresh Clot	2203.06	1.78
Fresh Clot	1500.17	1.83
Fresh Clot	2116.03	1.74
Fresh Clot	2200.00	1.82
Number of Observations	7.00	7.00
Maximum Value	3900.00	1.83
Minimum Value	1500.17	1.70
Co-efficient of Variation	32.47	2.60
Mean ± SEM	2309.89A±283.46*	1.78±0.017
4°C	1805.00	1.72
4°C	1156.00	1.80
4°C	1800.00	1.79
4°C	830.65	1.88
4°C	1368.40	1.80
4°C	1400.11	1.70
4°C	1795.12	1.75
4°C	2179.00	1.84
4°C	1120.00	1.76
4°C	1730.47	1.83
4°C	1264.36	1.70
Number of Observations	11.00	11.00
Maximum Value	2179.00	1.88
Minimum Value	830.65	1.70
Co-efficient of Variation	26.56	3.31
Mean ± SEM	1495.37B±119.76*	1.78±0.018
-21oC	1050.00	1.70
-21oC	550.01	1.80
-21oC	864.09	1.79
-21oC	842.00	1.80
-21oC	140.10	1.69
-21oC	1201.54	1.80
-21oC	1061.00	1.80
-21oC	329.75	1.66
-21oC	120.30	1.67
Number of Observations	9.00	9.00
Maximum Value	1201.54	1.80
Minimum Value	120.30	1.66
Co-efficient of Variation	60.22	3.62
Mean ± SEM	684.31C ±137.35*	1.75±0.02

(*)Significantly different at 5% level of significance.

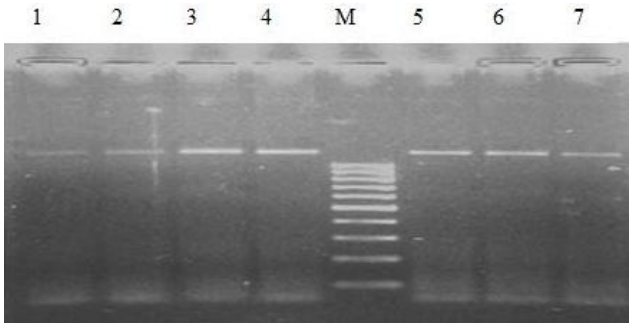

Fig.-1 : Genomic DNA samples of Ghraiyal

Lane M - 100 bp ladder
Lane 1, 2 & 7 - Samples stored at -21°C
Lane 3 & 4 - Fresh clots
Lane 5 & 6 - Samples stored at 4°C

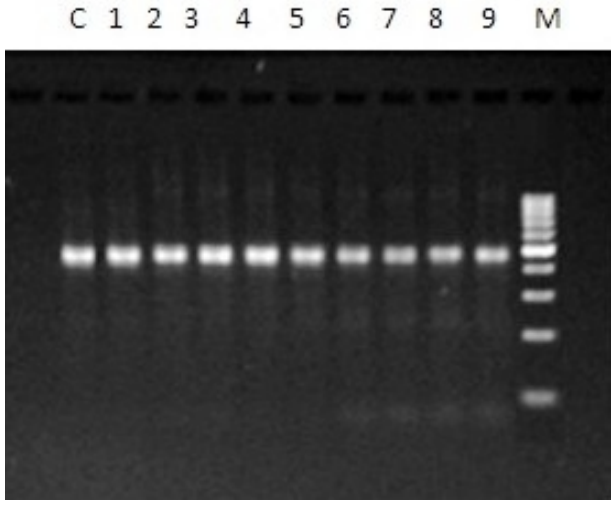

Fig.-2 : Amplified PCR product obtained from samples stored at different temperature lane

C - Positive control
Lane M - 100 bp ladder,
Lane 1-3—Samples from fresh clots
Lane 4-6—Samples stord at 4°C.
Lane 7-9—Samples stored at -21–C

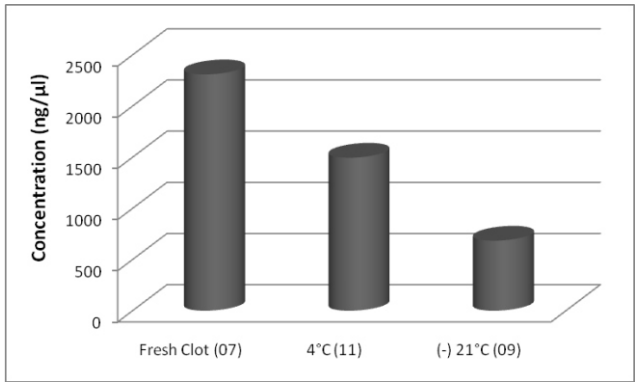

Fig.-3 : Clustered cylinder showing the mean concentration (ng/µl) of DNA in the blood clot

the supernatant is taken and the DNA is extracted twice with phenol: chloroform (1:1, v/v) and once with chloroform. The DNA is then precipitated by adding double amount of chilled absolute ethanol. It is then again centrifuged at 11000 rpm for 10 min at 4°C; the DNA pellets obtained are washed 2-3 times with freshly prepared 70 % ethanol and then dissolved in 100 µl 1X TE buffer and stored at -21°C till further use.

The DNA concentration was measured by Nano-drop spectrophotometer (ND-1000) and DNA purity was determined by the A260/A280 ratio. The integrity of the DNA samples was verified by 0.8% agarose gel electrophoresis after ethidium bromide staining under ultraviolet light.

PCR amplification : The samples were diluted to working concentration 30ng/µl and set up in a PCR reaction. The universal primers of mitochondrial 16S rRNA gene (4) were used for PCR amplification, with the following sequence, forward 5'-CGCCTGTTTAT CAAAAACAT-3' and reverse 5'- CTCCGGTTTGAA CTCAGATC-3'. A typical PCR cocktail consisted; DNA sample 3µl (30ng/µl concentration), PCR master mix 12.5 µl, nuclease free water 7.5 µl and forward primer and reverse primer 1 µl each with concentration of 10 p mole/µl. Total 25 µl of reaction mixture was briefly centrifuged at 1000 rpm to mix the contents and then subjected to PCR. The PCR cycle consisted of following steps, initial denaturation at 95°C for 5 min, denaturation at 94oC for 30 sec, annealing at 49.7°C for 30 sec, extension at 72°C for 1 min (40 cycles), and final extension at 72°C for 5 min. The products obtained were then subjected to 1.5 % agarose gel electrophoresis to check for amplification, band sizing was done with the help of 100 bp DNA ladder and Gel analyser 2010 software.

The obtained data was analyzed as per the standard methods5. Results were expressed as mean±SE. One way analysis of variance (CRD) was done to evaluate differences of yield and purity between DNA extracts. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The current protocol described above was successful in isolating good quality genomic DNA from all the three

categories of clotted blood samples, the concentration and purity of genomic DNA was assessed by OD at 260/280 for processed fresh ranging from 1500.17 ng/µl to 3900 ng/µl and 1.70 to 1.83; for 4°C ranging from 830.65 ng/µl to 2179.00 ng/µl and 1.70 to 1.88; and for -21oC ranging from 120.30 ng/µl to 1201.54 ng/µl; and 1.66 to 1.80 respectively (Table-1).

The yield of DNA concentration (ng/µl) for processed fresh samples and for stored samples at 4°C and -21°C (Fig. 3) showed significant differences ($P<0.05$) in present protocol. Thus it can be concluded that the protocol for extraction of DNA from stored sample at 4°C and -21°C could be efficiently done with the present protocol. However, the yield of DNA concentration (ng/µl) in stored samples at -21°C was significantly ($P<0.05$) lower as compare to processed fresh and stored samples at 4°C. The quality of DNA (OD 260/280) for processed fresh, stored at 4°C and -21°C samples was equally good. Statistically, it showed non-significance difference.

Assessment of quality on 0.8% gel electrophoresis showed in Figure-1 and 2 that the samples that were washed with freshly prepared 70% ethanol for at least three or more times showed a much better quality than those that were washed just twice, in some samples we used 3M sodium acetate for precipitating out the DNA but that increased the salt concentration and long streaks were seen on the gel, hence it is recommended to add only chilled absolute ethanol that instantaneously precipitates the DNA on addition. Besides low OD, all the above samples were successfully amplified by universal mitochondrial rRNA primers in a PCR reaction and we were able to obtain a 500 bp band in all samples (Fig. 2) while control having DNA obtained from unclotted blood samples from Ghraiyal, used to check whether DNA from clots amplifies the same or not.

CONCLUSION

This method of DNA isolation is efficient in extraction of good quality genomic DNA from leftover blood clots stored at either 4°C or -21°C. The yield of DNA was lower in samples stored at -21°C but it was sufficient to be amplified by PCR, this might have occurred due to longer storage time of samples kept at -21°C. All samples were positively amplified by PCR using universal primers, which shows that the method can be efficiently utilized for molecular characterization, forensic or genotyping studies.

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