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GENOMIC DNA EXTRACTION METHODS: A COMPARATIVE CASE STUDY WITH GRAM-NEGATIVE ORGANISMS

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Abstract. The cell wall structure of gram–negative bacteria is more complex than that of gram–positive bacteria because of the presence of two layers external to the cytoplasmic membrane. Purpose of this study is to compare the four different methods for genomic DNA extraction from three gram negative strains–*Escherichia coli, Klebsiella* species, and *Salmonella typhi.* The genomic DNA was extracted from gram–negative bacteria cultures using enzymatic lysis with Proteinase–K, chemical lysis with cetyl trimethyl ammonium bromide (CTAB), physical lysis with Triton X–100 and Beads beating methods. The yield and quality of DNA were compared by spectrophotometry and agarose gel electrophoresis. The genomic DNA purity ratio was found within 1.1–1.9 for all four methods. This study revealed that the chemical lysis with CTAB was the most efficient method for extracting genomic DNA from gram negative strains. In terms of chemical lysis with CTAB for *Salmonella typhi* and *Klebsiella* species the highest purity ratio were 1.9 and 1.8 respectively. However *Escherichia coli* also showed the highest purity ratio (1.7) for CTAB extraction. Enzymatic lysis with Proteinase–K was also very effective and purity ratio for *Salmonella typhi* and *Klebsiella* species were 1.8 and 1.7 respectively.

Keyword*:* Bead beating, CTAB, Genomic DNA, gram negative organism, Proteinase–K, Triton X–100

Introduction

Deoxyribonucleic acid (DNA) with its role as the repository of genetic information can arguably be considered the most important biomolecule in all available living systems and so as a part of the core architecture of life.

With the recent advancement, optimal sample processing protocols for diverse field of molecular biology require the release of DNA from an array of target organisms with equal efficiencies and without introducing DNA contamination.

The development of molecular biological methods i.e. DNA based method involving DNA extraction and Polymerase Chain Reaction (PCR) have also led to an array of new techniques that are not limited by the culturability of the microorganisms.

Broad–range application of bacterial DNA from clinical specimens with the aid of recent molecular technique has simultaneously proved useful for the diagnosis of various bacterial infections, especially during antimicrobial treatment of the patient [RANTAKOKKO–JALAVA and JALAVA, 2002]. Therefore, the extraction of DNA from natural environment has become a useful and an attractive alternative for the study of different microbial communities [AMANN *et al.,* 1995; BORNEMAN and HARTIN, 2000; HUGENHOLTZ *et al.*, 1998: PUTNOKY *et al.*, 2013].

Equally the isolation and purification of DNA from cells is considered as one of the most initial but key procedures in contemporary molecular biology.

Although a large number of methods have published for the extraction of total genomic DNA [STEFFAN *et al.,* 1988; ZHOU *et al.,* 1996] the most appropriate method for a specific application demands consideration of the issues like source, yield, purity and integrity.

The secrecy of a living organism sealed in the DNA must be secured, though most bacteria contain their genetic material in typically a single circular chromosome located in the cytoplasm in an irregularly shaped body called the

nucleoid, that is further jammed with intracellular protein, lipid and other materials [THANBICHLER *et al.*, 2005].

Even though the fundamental processes of DNA extraction from cells and tissues begin with sample lysis and the segregation of the nucleic acid away from contaminants, the number and diversity of these DNA extraction protocols used are quite miscellaneous [HERRICK *et al*., 1993; HOLBEN *et al*., 1988; KUSKE *et al.*, 1998; MORE *et al.*, 1994; OGRAM, 1987; PICARD *et al.*, 1992; ZHOU *et al.*, 1996].

But each protocol usually includes from one to all three of the basic elements: physical disruption, chemical lysis, and enzymatic lysis [MILLER *et al.*, 1999].

Two different physical disruption methods have been described: Triton X–100 extraction and Beads beating method, where Triton X–100 is a non–ionic detergent that can isolate the nuclear material with structural integrity [KUFF, 1964] and Bead beating is another popular cell breakage method [COURTOIS *et al.*, 2001; CULLEN and HIRSCH, 1998; MORE *et al.*, 1994; OGRAM, 1987; TEBBE and VAHJEN, 1993], where samples are supplemented with small zirconia/glass beads and then violently shaken to physically disrupt cells.

This is generally smaller and less sophisticated version though the size of the beads used, the period of milling, and the breakage buffer composition vary between protocols. The drawbacks to bead mill homogenization include the fact that larger amounts of contaminating humic acids are recovered when soil microorganisms are the sample [LEFF *et al.*, 1995; OGRAM, 1987] and also in some instances, the DNA can be sheared [LEFF *et al.*, 1995]. The chemical lysis procedures used in the method involve cetyltrimethylammonium bromide (CTAB).

It facilitates breakage and the subsequent deproteination in increased salt concentration [EDGCOMB *et al.*, 1999; PORTEOUS, 1991; RANJARD *et al.*, 1998; ZHOU *et al.*, 1996] and also can precipitate genomic DNA.

It is also popular because of its ability to remove polysaccharides from bacterial and plant preparations [AUSBEL *et al.*, 1998]. A final component of many DNA extraction techniques is enzymatic lysis. Lysozyme [BRUCE *et al.*, 1992; ERB and WAGNER–DOBLER, 1993; HERRICK *et al.*, 1993; TEBBE and VAHJEN, 1993; TSAI and OLSON, 1992], proteinase K [DEGRANGE AND BARDIN, 1995; TEBBE AND VAHJEN, 1993; ZHOU *et al.*, 1996] and achromopeptidase [DEGRANGE and BARDIN, 1995] have all been employed to promote cell lysis.

Particularly, proteinase K cleaves glycoprotiens and inactivates RNase and DNase in 0.5 to 1% SDS solution which has a particular pattern of effect on DNA extraction from both the gram–positive and gram–negative bacteria [SHAHRIAR *et al.*, 2011; KABIR *et al.*, 2006]. While the strengths and weaknesses of the various complete extraction and purification methods have been discussed previously, in general a more detailed comparison of individual elements of these methods for DNA extraction from gram–negative bacteria (*Escherichia coli, Klebsiella* species, *Salmonella typhi*) have been performed here. Meaningful comparisons are also confounded by the variable analytical procedures used to quantify DNA extraction efficiency and purity.

The objectives of this study were to compare the most common elements of DNA extraction and purification protocols for gram–negative bacteria and to use the information obtained to develop a comprehensive method for obtaining high yield of pure genomic DNA in a less labor–intensive way from variety of sample containing different quantities of organic matter.

Material and methods

Collection and analysis of samples

Blood, stool, rectal swab and pus specimens were collected from patients' specimens attending different diagnostic centers of Dhaka, Bangladesh.

Samples were screened for major gram–negative pathogens including *Escherichia coli, Klebsiella* species, and *Salmonella typhi.*

Isolation of microorganisms

Gram–negative organisms (*Escherichia coli, Klebsiella* species, and

Salmonella typhi) were collected from varieties of patients' specimens.

These organisms are pathogenic in nature and wide spread in natural environment. After collection, these organisms were cultured and allowed to grow in nutrient agar media for 24 hrs at 37°C temperature. Afterward, organisms in nutrient broth were placed in a metabolic shaker at 37°C with 100 rpm for 24 hrs. The cell pellets were separated from the media by centrifugation for DNA extraction.

Genomic DNA extraction

Four different methods were followed for genomic DNA extraction– Triton X–100 extraction methods, Bead beating methods, enzymatic treatment with Proteinase–K and chemical lysis with cetyl trimethyl ammonium bromide (CTAB).

Triton X–100 extraction method:

The cell pellets were washed with 1 mL TE buffer and re–suspended in 270 µL of STET solution (pH 8.0) containing 8% (w/v) sucrose, 5% (v/v) Triton X–100, 50 mM EDTA and 50 mM Tris–HCl. The cell suspensions were treated with 15 µL of chloroform in a shaker for 5 mins.

After this treatment, the suspensions were incubated for 30 mins at 37°C upon addition of 30 µL of lysozyme (20µg/mL).

At the end of this incubation 100 µL 10% SDS was added to the suspensions and incubated for another 60 minutes at 65°C. Afterward 100 µL of 5 M NaCl was added and the solution was washed three times with the same volume of chloroform. The chromosomal DNA was precipitated from the aqueous solution by addition of the same volume of 100% isopropanol which was further collected by centrifugation at 12,000 g for 20 min at 4°C. Then the extracted DNA was washed three times with 70% ethanol, finally resolved in 200µL TE buffer and stored at –20^{o}C.

Bead Beating Method:

750 µl of potassium phosphate buffer and 250 µl of 1% SDS were added in each eppendorf tube containing the cell pellets. Subsequently 1 mm diameter sized glass beads were added to each tube. Then the total solution was centrifuged at 1,500 g for 15 mins at 10°C. The supernatant was collected into new Eppendorf tube and immediately 9 µl of 0.5 M EDTA and 1 µL of 0.5 M potassium acetate were added.

Then the suspension were kept in ice for 20 minutes and then centrifuged at 15,000 g for 5 minutes at 4°C.

The supernatant was transferred into new tubes. Then the DNA in the supernatant fraction was precipitated out with equal volume of 100% isopropanol (16 µL) by keeping it for 1 hour. DNA was then recovered by centrifugation at 15,000 g for 1 minute at 4°C. Finally the pellets were re–suspended using high molecular grade water.

Enzymatic treatment with Proteinase–K:

At first cell pellets in the eppendorf tubes were re–suspended in 567 µL of TE buffer. Then 30 µL of 10% SDS was added and properly mixed followed by an addition of 3 µL of Proteinase–K.

Afterward 100 µL of 5 M NaCl and 80 µL of CTAB/NaCl solution were added and mixed thoroughly and the suspension was incubated for next 10 min at 65°C. After the incubation, equal volume (180 µL) of chloroform/isoamyl alcohol (24:1) was added to this suspension which further centrifuged at 12,000 g for 4 min.

The supernatant was transferred to fresh Eppendorf tube and equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added followed by centrifugation at 12,000 g for 4 min.

The supernatant was collected and 0.6 volume of 100% isopropanol was added and stored at –20°C for 24 hrs. DNA pellets were then obtained by centrifuging for 4 min at 12,000 g and re–suspended with 100 µL of TE buffer to eventually store at –20°C.

Chemical lysis with cetyl trimethyl ammonium bromide (CTAB):

250 µL of CTAB buffer was added to the sample pellets in each Eppendorf tube. The suspension was homogeneously grinned and 2.5 µL of Proteinase–K (20 mg/mL) was added followed by overnight incubation at 50°C.

The next day 2 volumes of chloroform/isoamylalcohol (24:1) were mixed thoroughly with the suspension which was further centrifuged for 5 min at 12,000 g. The upper aqueous phase was collected from where nucleic acids were precipitated by the addition of 2/3 volume of ice–cold 100% isopropanol.

Precipitated DNA were kept at –20°C for 1 hr and then centrifuged for 30 min at 12,000 g. The pellet was collected and washed with 1 mL of 70% ethanol.

Later the pellets were air–dried for 10 min, re–suspended in 50–100 µL of sterile distilled water and stored at –20°C.

Quantitative analysis of extracted DNA

In order to know the DNA concentration total extracted DNA was quantified using UV / VIS Spectroscopy (JASCO, Japan) at 260 nm and converted to concentration considering absorption of 1 OD (A) is equivalent to approximately 50 µg/mL ssDNA [SAMBROOK *et al.*, 1989: RASHED and BUTNARIU, 2014a]. Purity ratio (A260:A280) was also determined by determining the absorbance value at 280 nm.

Qualitative analysis of extracted DNA

Gel electrophoresis was carried out for the qualitative analysis of extracted DNA. The value of the extracted DNA (absence of degradation) was estimated based on the size on the DNA fragments or the relative position of the DNA grades after electrophoresis of aliquots of the DNA solution on 1% of agarose gel.

Photographs of stained gel were taken directly on a UV transilluminator by gel documentation system.

Results and discussion

Quantitative estimation of total extracted genomic DNA

Absorbance value at 260 nm was considered for quantitative estimation of DNA.

Table 1.

Total yield and purity ratio of the genomic DNA extracted by different methods from different samples of Gram negative strains

Name of the Method	Name of the organisms	Samples	Concentration (µg/mL)	Purity Ratio (260/280)
Triton X–100	*Escherichia coli*	E1	1.12	1.4
		E2	0.082	1.3
	Klebsiella species	K1	0.57	1.6
		K2	0.5	1.3
	Salmonella typhi	S1	0.047	1.3
		S2	0.025	1.4
Bead Beating	*Escherichia coli*	E3	0.075	1.4
		E4	0.05	1.5
	Klebsiella species	K3	1.2	1.5
		K4	2.15	1.3
	Salmonella typhi	S3	2.7	1.6
		S4	1.8	1.4
Proteinase–K	*Escherichia coli*	E5	1.5	1.1
		E6	1.2	1.5
	Klebsiella species	K5	2.5	1.7
		K6	1.4	1.6
	Salmonella typhi	S5	0.077	1.5
		S6	0.047	1.8
CTAB	*Escherichia coli*	E7	2.02	1.5
		E8	0.06	1.7
	Klebsiella species	K7	1.62	1.8
		K8	1.7	1.7
	Salmonella typhi	S7	2.05	1.4
		S8	1.02	1.9

The absorbance of 1 OD (A) is equivalent to approximately 50 µg/mL ds DNA, approximately 33 µg/mL ssDNA, 40 µg/mL RNA or approximately 30 µg/mL for oligonucleotides [ZHOU *et al.*, 1996: RASHED and BUTNARIU, 2014b]. Extraction protocols were

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carried out using physical lysis with Triton X–100, Bead beating method, enzymatic lysis with Proteinase–K and chemical lysis with CTAB. Among these techniques the highest DNA concentration 2.7 µg/ µL was found in beads beating methods for the sample S3 of *Salmonella typhi* whereas the lowest concentration was found in Triton X–100 method for sample S2 of same strain (Table 1).

Determination of Purity Ratio

Purity of DNA can be recognized by the calculation of "purity ratio". The ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid. Pure DNA should have ratio of approximately from 1.6–1.8.

The highest purity ratio (1.9) was obtained in sample S8 of CTAB method and lowest value (1.1) was seen in sample E5 of Proteinase–K method (Table 1). A comparative study has been done to observe the purity ratio of the extracted DNA samples by different methods. The highest quality DNA was obtained by CTAB method in different samples (Figure 1).

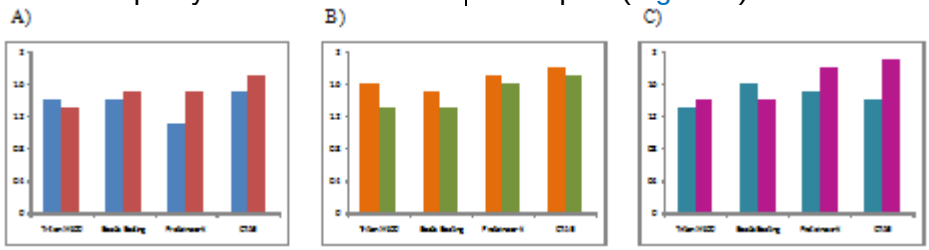

Figure 1. Comparative study of the purity ratio of the extracted bacterial genomic DNA obtained from different samples of A) *Escherichia coli*, B) *Klebsiella* species, and C) *Salmonella typhi* by four different extraction protocols.

The quality of extracted bacterial genomic DNA obtained from three gram negative strains were compared for four different extraction protocols by gel electrophoresis.

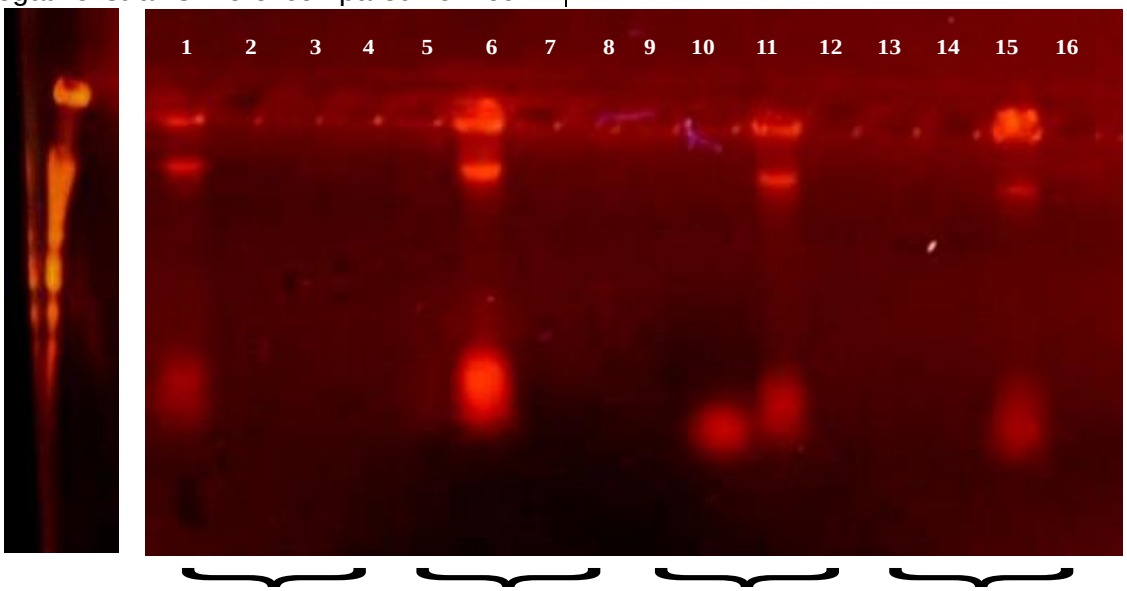

Figure 2. Agarose gel (1%) electrophoresis of total extracted genomic DNA from two samples each of *Klebsiella* species and of *Salmonella typhi.* For physical lysis with Triton X–100 extraction of K1, K2, S1 and S2 show genomic DNA bands in lane 1, 2, 3, and 4 subsequently. However, for Bead beating methods K3, K4, S3 and S4 samples of *Klebsiella* species and *Salmonella typhi* show genomic DNA bands in lane 5, 6, 7, and 8 accordingly. For the same samples of *Klebsiella* species and *Salmonella typhi* DNA bands in lane 9, 10, 11, and 12 were found in terms of Proteinase–K method. For chemical lysis with CTAB K7, K8, S7 and S8 samples show genomic DNA bands in lane 13, 14, 15, and 16 respectively. The first separate lane contain DNA marker.

For *Klebsiella* species and *Salmonella typhi* high molecular weight intact genomic DNA was obtained by using all four techniques (Figure 2).

In terms of Proteinase–K extraction method we observed two high molecular weight intact DNA bands for K6 and S5 samples.

For *Escherichia coli* high molecular weight intact DNA bands were observed for almost all three techniques except in physical lysis with Triton X–100 for sample E1 and E2.

In terms of quality of the extracted genomic DNA we found that all the three gram negative strains gave high molecular weight intact genomic DNA band in case of chemical lysis with CTAB.

We also found intact DNA in enzymatic lysis with Proteinase–K, physical lysis with Triton X–100 extraction and beads beating methods.

According to this study, chemical lysis with CTAB has been found to be best method and the highest quality of isolated DNA was found with this method for all the three gram negative strains of this experiment.

Although DNA with high purity ratio for all the techniques was observed, but due to degradation and RNA contamination it was not possible to get the intact DNA for all techniques.

This problem might be resolved by using RNAse treatment.

On the contrary, our study revealed that for the quantity extraction of genomic DNA, chemical lysis with CTAB was observed as the most effective technique.

This is because of the highest yields of genomic DNA and highest average purity ratio was found for this technique.

In enzymatic lysis with Proteinase–K the yield of genomic DNA was also high and consistent for the strains.

From this study it can be said that the cell wall of gram negative organism is very susceptible to the chemical lysis with CTAB. At the same time physical lysis with Triton X–100 is comparatively less effective process among the four techniques on the basis of value of purity ratio.

Conclusions

In many cases the traditional DNA isolation techniques can give better yield compared to modern DNA isolation kits available in the market.

Moreover, these methods are cost effective and easily available.

Further research can be done to establish and ensure the current observation of this study.

Competing interests

The authors declare that they have no competing interests.

Author's Contribution

Prof. Mohiuddin Ahmed Bhuiyan and Mohammad Shahriar designed the experiment. Sanjida Jahan, Shahida Akter, and Sayeda Fahmee Chowdhury carried the experiment and performed the data analysis. Sanjida Jahan drafted the manuscript and Prof. Bhuiyan corrected it.

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