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ORIGINAL ARTICLE

Boiling, Freezing-thawing and Simple Chemical Extraction Methods are Comparable to Costly Kit-based Bacterial DNA Extraction Methods as Evidenced by Conventional and Real-time PCR

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Key words:

Boiling; Freezing-thawing; SDS; Tween; DNA; PCR

ABSTRACT

Background: *Bacterial DNA extraction is a preliminary step for PCR studies carried out on bacteria for diagnosis and detection of antimicrobial resistance genes.* ***Objectives:*** *Testing efficiency of two physical methods (boiling and freezing-thawing) and two chemical methods (SDS and tween based methods) for extraction of bacterial DNA suitable for conventional as well as real time PCRs in comparison to a costly ready to use kit method.* ***Methodology:*** *Escherichia coli ATCC 25922 was submitted for five DNA extraction methods including QIAamp ready kit, boiling, freezing-thawing in TE, SDS method, and tween method. DNA purification by ethanol precipitation and wash was carried out after tested physical and chemical methods. The extracted DNAs were assessed by Nanodrop spectrophotometer, conventional PCR followed by Uviband quantification, and semiquantitative SYBR green based RT- PCR.* ***Results:*** *Nanodrop assessment showed that QIAamp kit and tween method produced the highest DNA yields; 78.1 ng/µl and 49.72 ng/µl, respectively, while SDS method gave the lowest. Both QIAamp kit and tween methods produced comparable DNA in concentration and purity. All tested methods extracted DNA amplifiable by PCR. In conventional PCR, the concentrations ranged from 287.31 to330.46 ng/5µl for boiling and QIAamp kit, respectively. In RT-PCR, the highest CT values were obtained by OIAmp kit, followed by tween, freezing-thawing in TE, SDS, and boiling methods with narrow range of variation (from 30.80 to 31.85). No significant statistical differences were detected between tested methods in the results of PCRs either conventional or real time.* ***Conclusion:*** *Boiling in water, freezing-thawing in TE, tween and SDS based methods followed by DNA precipitation provide rapid, effective, less laborious and truly cost-effective methods for extraction of bacterial DNA suitable for PCR studies.*

INTRODUCTION

Molecular biological methods have become corner stones in the field of medical bacteriology. Indeed, these methods comprise wide range of techniques including, for example, polymerase chain reaction (PCR); either conventional or real time, restriction fragment length polymorphism (RFLP), southern blotting and sequencing, and provide solutions to many problems faced in the clinical microbiology and infectious diseases practices especially in diagnosis and tracing of infectious diseases[1-3].

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The application of such methods shorten the time needed for routine identification of bacteria using ordinary culture based methods[4] and provide effective tools for identification of atypical organisms[5] as well as studying the antimicrobial susceptibility patterns and detection of resistance genes [6-8]. Added to this, the accurate tracing and investigations of outbreaks of foodborne and nosocomial infections [9-11].

The DNA extraction from bacteria is a preliminary and fundamental step for all subsequent molecular techniques carried out on these bacteria and can be carried out using numerous methods including; physical methods like boiling, freezing-thawing, glass bead beating, and sonication [12-14], chemical methods with use of phenol-chloroform[15] and different lysis buffers utilizing detergents like sodium dodecyl sulfate (SDS) and tween[16-18], ready to use extraction kits [19], and automated extraction systems [20-22]. All of these methods differ in the quantity and quality of the extracted DNA and consequently in the outcomes of the subsequent

molecular processes. So that, selection of an effective, simple, reproducible, cheap method is truly needed in field of molecular biology.

This study aimed at testing efficiency of some simple DNA extraction methods and suitability of DNA extracted by them for conventional as well as real time PCRs in comparison to relatively costly ready to use kit methods. The study compared five different methods for DNA extraction from bacteria including simple physical (boiling and freezing-thawing), chemical (sodium dodecyl sulfate (SDS) and tween based) and a kit- based method (QIAamp DNA Mini kit). In this comparison, both conventional and real time PCR assays were applied for evaluation of the extracted DNA. *Escehericha coli* was selected as a test organism as it is one of the most common bacteria encountered in the clinical microbiology practice.

METHODOLOGY

Bacterial strain

Escherichia coli ATCC 25922(Becton Dickinson, France)was used as a test strain. It was subcultured in tryptone soya broth (TSB) (Himedia, Mumbai, India) overnight at 37°C with shaking, then dispensed in 1 ml volumes in sterile tubes, the turbidity of these bacterial suspensions were adjusted approximately to 1McFarland as measured by spectrophotometer at 600 nm which equal approximately to 3×10^{8} CFU/ml.

DNA extraction

The bacterial suspensions were centrifuged at 13,000 rpm for 10 minutes, the supernatants were discarded and the pellets were washed three times using phosphate buffered saline (PBS), then the pellets were subjected to the following extraction methods: A) QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 200 µl of TE buffer and stored at -20°C till use. B) Boiling in sterile RNase, DNase free water as modification of method of da Silva et al.[23]; briefly, the bacterial pellet was resuspended in 200 µl of sterile water, vortexed well and boiled at 100°C for 20 minutes, then let to cool to room temperature. After that, it was vortexed well and centrifuged at 13,000 rpm for 20 minutes, and the supernatant was transferred to new sterile 1.5 ml tube.C) Alternating freezing and thawing using TE buffer (10mM Tis-HCl, 1 mM EDTA (pH 8) as previously done byYang et al.[24] with few modifications; briefly, the bacterial pellet was resuspended in 200 µl of TE buffer, vortexed and then heated to100°C for 2 minutes then exposed to three cycles of freezing at -70°C for 4 minutes and thawing by boiling at 100°C for 3 minutes. Afterwards, the tubes were vortexed and centrifuged at13,000 rpm for 20 minutes and the supernatant was transferred to new sterile 1.5 ml tubes.D) SDS-based extraction using lysis buffer (10mM Tis-HCl, 1 mM EDTA (pH 8), 0.5% SDS, 200 µg/ml proteinase K)as mentioned by Goldenberger[16];the pellets were resuspended in 200 µl of this buffer, vortexed and incubated at 56°C for 3 hours, then heated at 95°C for 10 minutes to inactivate the proteinase K. Afterwards, the samples were centrifuged at13,000 rpm for 20 minutes and the supernatant was transferred to new sterile 1.5 ml tubes. E) Tween-based extraction using lysis buffer (10mM Tis-HCl, 1 mM EDTA (pH 8), 0.5% Tween, 200 µg/ml proteinase K) as mentioned previously [25] and exposed to the same processing as the SDS method.

Sodium acetate or sodium chloride treatment and ethanol precipitation

All of the above mentioned physical and chemical methods were followed by DNA purification through DNA ethanol precipitation and wash according to Green and Sambrook[26], briefly, twenty microliters of sodium acetate (3 M) were added to each sample obtained from boiling, freezing-thawing, and tween methods, while 20 µl of sodium chloride (2 M)were added to samples of SDS method (to help to remove the traces of the SDS). The samples were mixed gently and let at room temperature for 30 minutes, and then 600 µl of absolute ethanol were added to each sample, mixed gently then kept at –20°C overnight. The samples were then centrifuged with discarding of the supernatant and subsequently the precipitated DNA was washed by 70% ethanol and left to air dry, then eluted in 200 µl of TE buffer and stored at -20°C till further processing.

To accurately compare between the used five methods, the initial bacterial suspension samples used for extraction were equal in volume(1 ml)and of approximately equal turbidity as measured spectrophotometrically (1 McFarland).The extractions, for all methods, were made from 3 samples at the same time and the extracted DNAs were eluted in equal volumes (200 µl)of sterile TE buffer. To guarantee the reproducibility, the extractions were repeated three times on three different occasions.

Nanodrop spectrophotometry

All of the extracted DNAs were submitted for spectrophotometric assessment using the NanoDrop 2000 spectrophotometer (ThermoScientific, USA). For each sample, determination of OD_{260}, OD_{280}, OD_{260}:OD_{280} ratio and DNA concentration in nanograms/µl was carried out.

Conventional PCR

According to Nadkarni et al. [27], all extracted DNAs were used as templates for PCR amplifications targeting a region in 16 rDNA of the tested organism with production of amplicon of 466 bp size by use of two primers; forward primer:

5'-TCCTACGGGAGGCAGCAGT-3' and reverse primer:

5'- GGACTACCAGGGTATCTAATCCTGTT-3' (TIB MOLBIOL, Germany). The reactions were carried out

in volumes of 50 µl consisting of 25 µl of 2× Go Taq Green Master Mix (Promega, Madison, USA), 0.4 µM of each primer,10 µl of extracted DNA, completed to a total volume of 50 µl with sterile RNase and DNase free water. The reactions were carried out using a thermal cycler (Eppendorf Master cycler gradient, Germany) under cycling conditions: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 40 sec, 55°C for 45 sec, and 72°C for 45 sec, followed by an extension step of 72°C for 6 min.

Gel electrophoresis

The amplified PCR products were run in gel electrophoresis of 1.5% agarose, in 1× TAE buffer, 80 V for 1.5 hour. Exact Mark 100 bp DNA ladder (1st Base, Singapore) was used as a marker. The gels were stained by ethidium bromide and photographed by Uvitec gel documentation and analysis system (Cambridge, UK). The PCR products (bands) were analyzed and quantified using Uviband software provided in this documentation system.

Real time PCR (RT-PCR)

All extracted DNAs were submitted for SYBR green based real time PCR. The amplified products were measured in semiquantitative manner depending on the determination of fluorescence threshold cycle (CT), as the earlier the appearance of fluorescence the higher the concentration of the amplified product. Both previous primers mentioned above were utilized to target 16S rDNA of the tested bacteria[27]. The amplifications were carried out in 25 µl volumes consisting of 12.5 µl of Fast Start SYBR Green Master kit (Roche Diagnostics Corporation, IN, USA), 0.4 µM of each primer, 7 µl of extracted DNA and completed to total volume of 25 µl by PCR grade water. Negative control reactions were run with replacement of the DNA by sterile RNase and DNase free water. The reactions were performed using Smart cycler machine (Cepheid). The cycling conditions included pre-incubation at 95°C for 10 min, and 45 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The value of fluorescence threshold cycle (CT) was determined for each amplification. The PCRs were repeated three times on different three occasions.

Statistical analysis

The results of different used DNA extraction methods were expressed in forms of means and standard deviations for each of evaluating tests (i.e. DNA concentration and purity ratio by Nanodrop spectrophotometer, concentration of conventional PCR products and CT number values of RT-PCR). The comparison between these methods was carried out using one-way ANOVA with application of Post-hocmultiple comparisons Tukey test. The significant statistical difference was considered when *P* value < 0.05.The IBM SPSS Statistics 20 program (IBM corporation, New York, USA) was used for data entry and analysis.

RESULTS

Spectrophotometric assessment

The DNAs extracted from the five methods were assessed by NanoDrop 2000 spectrophotometer and the results are shown in table 1. The yields of DNA were different between methods, as the highest yields were 78.1ng/µl and 49.72ng/µl and obtained by QIAamp kit and tween method, respectively, while SDS method gave the lowest concentration. The purity of the produced DNA was assessed by determination of the 260/280 ratio, which when equal 1.8-2 it indicates pure DNA[28]. By comparing this ratio between used methods, both QIAamp kit and tween methods produced the purest DNAs. The extracted DNAs by boiling in water and freezing-thawing in TE were approximately of same purity. The tested five methods showed overall significant statistical difference in the concentration and purity of the extracted DNA and this difference, as proved by Post-hock multiple comparison testing,was mainly between the QIAamp kit method and other methods except tween method. Moreover, boiling, freezing-thawing, SDS and tween methods showed no significant statistical difference.

Table 1: Assessment of DNA extracted by the five methods by NanoDrop 2000 spectrophotometer.

Method	Concentration(ng/µl[a])		Purity (260/280 ratio)	
	Mean	SD	Mean	SD
QIAamp kit	78.1	18.20	1. 89	0.17
SDS method	31.6	3.03	2.31	0.16
Freezing-thawing in TE	38.3	6.75	1.62	0.30
Boiling in water	35. 37	6.86	1.47	0.12
Tween method	49.72	7.81	1.92	0.19
P value	0.002		0.005	

Values shown are the means and standard deviations (SD) of three replicates for all tested methods.[a] microliter of the extracted DNA. Post-hoc multiple comparisons revealed significant statistical differences only between QIAamp kit method and: boiling, freezing-thawing in TE and SDS methods.

Conventional PCR assessment

The PCRs carried out on all DNA extracted from all methods were positive for all samples and produced amplified products of the same size of approximately 466 bp as shown in figure 1.The captured gel photos were assessed and analyzed by Uviband software of Uvitec gel documentation and analysis system (Cambridge, UK) to calculate the concentration of the amplified products. To determine the concentration by this software, all of the tested bands (PCR products) of DNAs extracted by the five methods were quantified in ng/5 µl of the PCR products in comparison to the molecular DNA marker (ExactMark 100 bp DNA ladder) that was taken as standard reference lane. The results are shown in table 2. The highest concentration was obtained by QIAamp kit method followed by tween method, then freezing and thawing in TE, then SDS method, while the least yield was obtained from the boiling in water. No significant statistical differences were detected between tested methods.

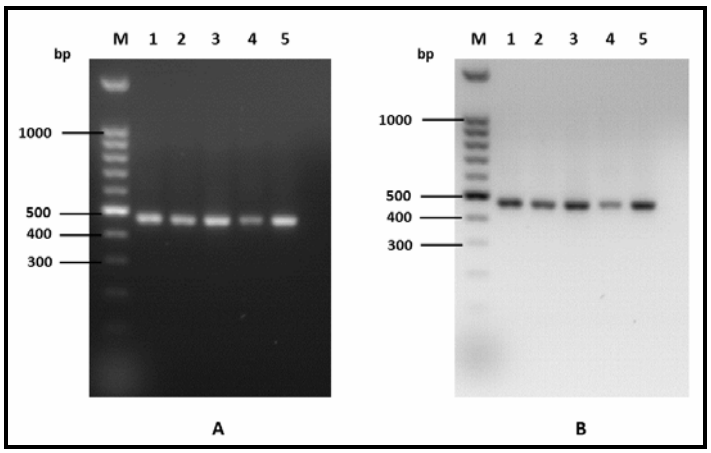

Fig. 1. Gel electrophoresis of the PCR amplified products of the five DNA extraction methods. A) Positive photo, B) Negative photo. Lanes: M, Molecular DNA marker [ExactMark 100 bp DNA ladder (1st Base, Singapore)]; 1, QIAamp method; 2, SDS method; 3, freezing-thawing in TE; 4, boiling in water; 5, tween method. All bands have the same sizes (approximately 466 bp) but with little differences in intensities (denoting differences in concentration). Measuring the concentration of the PCR bands using Uviband analysis software revealed that QIAamp kit and tween methods gave the highest concentrations while boiling gave the least concentration.

Assessment by real-time PCR (RT-PCR)

All DNA samples extracted by the five methods were submitted for semiquantitative assessment by determining the fluorescence threshold cycle (CT) using SYBR Green based real-time PCR. The earlier CT, the higher the concentration of the DNA in the sample. This assessment was carried out in three runs on three different times and the means of CT values of the three runs were determined for each sample and the results are expressed in table 2 for comparison between extraction methods. All DNAs extracted by all tested five methods gave positive RT-PCR. The highest CT values were found with DNA obtained byOIAmp kit, followed by tween, freezing-thawing in TE, SDS, and boiling methods. As shown in table 2, the range of the all CT values of tested five extraction methods was obviously narrow (from 30.80 to 31.85). The statistical analysis of these values revealed no significant statistical differences between tested methods.

Table 2: Assessment of the DNA extracted by methods used in the study using conventional and real time PCRs

Method	Uviband [a] analysis (ng/5µl [b])		Real-time PCR(CT value)	
	Mean	SD	Mean	SD
QIAamp kit	330.46	55.84	30.80	0.50
SDS method	299.83	50.66	31.62	1.65
Freezing-thawing in TE	313.31	52.94	31.50	0.69
Boiling in water	287.31	48.55	31.85	0.54
Tween method	317.19	53.59	31.35	0.84
P value	0.805		0.773	

Values presented are the means and standard deviations (SD) of three replicates for all tested methods. [a]Uviband analysis of the conventional PCR products using Uviband software of Uvitec gel documentation and analysis system.[b]5 microliters of the amplified PCR products (the amount loaded in the gel wells during the electrophoresis process either from the DNA ladder or amplified samples). CT is the fluorescence threshold cycle.

DISCUSSION

The current study compared five DNA extraction methods: two simple physical (boiling in water and freezing-thawing in TE) and 2 simple chemical (SDS and tween based methods) and one kit based method (QIAamp), which was taken as gold standard, to investigate the efficacy of these simple methods in extraction of bacterial DNA suitable for PCR amplification.

In comparison to previous studies[16, 23-25],some modifications were performed in this study including increasing incubation times of boiling, increasing the boiling temperature to 100 °C, increasing number of freezing-thawing cycles and thawing at 100 °C instead of room temperature, and use of the supernatant of bacterial lysate after centrifugation, added to this, post extraction DNA purification through adding sodium acetate or sodium chloride (in SDS method) and alcohol precipitation after all tested physical and chemical methods.

As regards the concentration and purity as measured by Nanodrop spectrophotometry for the extracted DNA, the QIAamp produced significantly pure DNA in comparison with freezing-thawing, boiling and SDS methods. On the other hand, tween method produced DNA comparable in concentration and purity to that extracted by QIAamp kit. The high purity of the QIAamp kit DNA is mainly due to column purification step, while the decreased purity of currently tested physical and chemical methods could be explained by presence of bacterial cell debris, RNA and traces of the chemicals used in extractionthat may remain in the lysate even after alcohol DNA precipitation and wash.

Both tween and SDS methods utilized in this study are considered enzymatically-based as they utilized proteinase K and such methods although the disadvantage of consuming relatively long time (around three hours), they were found to be simple and less laborious for extraction of bacterial DNA when compared to ready kit methods[25].

The tween method has a advantage over SDS method which is the absence of interference of tween traces with PCR amplification, whereas SDS traces, even in very low concentrations (0.01%),can interfere with the efficiency of subsequent PCR as it may cause denaturation of Taq. polymerase[16, 29], so that, treatment of the extracted DNA byNaCl and DNA precipitation, which were performed in this study, are crucial for removal of SDS from the resultant lysate to guarantee an effective PCR amplification.

One of the most important features of the extracted DNA is its suitability to perform PCR which is considered nowadays as a corner stone diagnostic tool in field of clinical microbiology[30], all methods tested in the current study succeeded to produce DNAs amplifiable by both conventional and real time PCRs indicating absence of inhibitors of PCR amplification. The absence of these inhibitors could be due to DNA purification carried out in this study by application of post-extraction alcohol precipitation and washing.This was more obvious with the SDS method as its DNA was amplified successfully in our study, in contrast to Aldous et al. who found that SDS extraction only without purification produced DNA not amplifiable by real time PCR [29].

In PCR-based quantitation either by conventional PCR using Uviband analysis software or RT-PCR by determining the fluorescence CT, our results revealed narrow range of variation between all tested five methods in the quantity of the amplified DNA and non significant differences neither in the concentrations of the conventional PCR amplicons or CT values of the RT-PCR indicating that tested two physical and two chemical methods are comparable to the gold standard QIAamp kit based method in the yield of the extracted DNA. These results agreed with those of Peng et al. who found that direct boiling gave comparable results to Qiagen and other commercial kits in extracting fecal microbiome suitable for sequencing of 16S rRNA tags[31].

In conclusion, although the tested physical and chemical methods produced DNA of decreased purity in comparison with the QIAamp kit, these methods produced good quality DNA that was amplified successfully by both conventional and real time PCR, indicating that boiling in water, freezing-thawing in TE followed by DNA precipitationcan provide rapid, effective and truly cheap methods for extraction of bacterial DNA. Moreover tween and SDS based methods providealternative cost-effective and less laborious DNA extraction methods.

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