

## Original Article

# Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques

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## ABSTRACT

**Objective:** To evaluate the efficacy of two simple methods involving use of heat for extraction of bacterial deoxyribonucleic acid (DNA) be used in molecular techniques like polymerase chain reaction (PCR), restriction fragments length polymorphism (RFLP) and DNA sequencing and compare them with DNA extraction using commercial kits.

**Design:** DNA extraction by improved alternative methods and commercial kit.

**Setting:** Microbiology Research Laboratory, Faculty of Allied Health Sciences, Kuwait University, Kuwait

**Material:** Forty isolates of *Klebsiella pneumoniae*

**Intervention:** DNA was extracted from isolates by either boiling for 10 minutes or microwave

irradiation for 10 seconds. For comparison, DNA was also extracted using a commercial kit. All extracted DNA samples were analyzed by PCR, RFLP and / or DNA sequencing of TEM and SHV genes of the bacteria.

**Main Outcome Measures:** Successful extraction of DNA

**Results:** PCR, RFLP and DNA sequencing gave the expected results in all the DNA samples extracted by all the three methods (boiling, microwave irradiation and the commercial kit). The results were qualitatively equivalent in all methods.

**Conclusion:** Heat may be used to extract DNA from *K. pneumoniae* which can be utilized successfully in performing PCR, RFL and DNA sequencing.

KEY WORDS: bacteria DNA extraction, DNA sequencing, PCR, RFLP

## INTRODUCTION

The science of molecular biology has become an integral part of all medical research fields including bacteriology. Techniques including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), hybridization techniques and DNA sequencing are being extensively used in identification and classification of different bacterial species and subspecies. In fact, many bacterial strains are now classified based solely on molecular characteristics<sup>[1-2]</sup>. Molecular techniques in bacteriology usually start with bacterial DNA extraction and purification. A large number of DNA extraction methods (performed manually or by automation) have been and are still being developed, each of which has its own advantages and

disadvantages. Many of these methods are based on the traditional phenol-chloroform extraction method, which needs a variable number of reagents and equipment<sup>[3-4]</sup>. Moreover, several trials have been made to simplify the procedure for bacterial DNA extraction and purification. These methods tried to break the cells and release the DNA using certain lysing agents containing different chemicals like lysosyme, proteinase K, TWEEN20, sodium hydroxide/sodium dodecyl sulfate, guanidine isothiocyanate, and Triton X-100<sup>[5-14]</sup>. In addition to chemical agents, physical factors have also been attempted including heating, cooling, freezing, microwave irradiation, beads beating, magnetic field capturing, binding to glass beads, the use of ultrasound waves and passing through heat-exchanger coils and nylon filters<sup>[5-18]</sup>.

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Many have also used combinations of chemical and physical methods<sup>[5-19]</sup>. Still, most of these methods are laborious, time consuming and costly. In the last two decades, many commercial kits have been developed to extract bacterial DNA using simpler steps and a shorter time frame. Although they made the DNA extraction process quicker, such methods are costly and require several steps and reagents, and sometimes special equipment, to obtain the target DNA<sup>[14,20]</sup>.

In this study, the authors have tried two very simple methods that may be used to extract bacterial DNA using heat only in a very simple manner. Using heat for bacterial DNA extraction is not new. High temperature exposure is known to cause damage to cell membranes and cell walls<sup>[14,16,20-22]</sup>. Jose and Brahmadathan reported that heating at 94 °C for two minutes was enough to denature cell walls<sup>[16]</sup>. Low temperatures were also observed to destroy cell walls and membranes. Freezing induces crystallization of water inside cells which leads to destruction of cytoplasmic structures<sup>[12,16,20]</sup>. In fact, Tell *et al* used cycles of freezing and thawing to obtain bacterial DNA<sup>[12]</sup>. In practice, heating bacterial material for DNA extraction purposes was performed by boiling in a water bath or on hot blocks, or using microwave ovens<sup>[5-16]</sup>. Microwaves can cause many different biological effects; these are mainly due to the heating process (thermal effects) but there are also athermal effects on cellular material, which were thought to be due to acceleration and collision of ions with other molecules, partitioning of ions, or altering the polarity of molecules in alternating electric fields<sup>[22-24]</sup>. In this study, the use of heat has been improved in two simplified ways to extract DNA from bacteria. To assess the suitability of the extracted DNA for performing molecular biology techniques, the extracted bacterial DNA was processed by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and DNA sequencing. For comparison, a commercial DNA extraction kit was also used. These two methods, as well as the commercial kit, were tried on *Klebsiella pneumoniae* isolates harboring extended spectrum  $\beta$ -lactamase (ESBL). ESBLs are mainly derived from TEM, SHV or CTX-M  $\beta$ -lactamases that have mutated to expand their spectrum of activity to include third generation cephalosporins<sup>[1,25]</sup>. Although they were first reported in *Klebsiella species*<sup>[26]</sup>, ESBLs are now also commonly found in *Escherichia coli* and they have also been found in other species of *Enterobacteriaceae*<sup>[1]</sup>. To date, more than 130 TEM and more than 104 SHV derivatives have been found<sup>[1,2,25]</sup>.

## MATERIAL AND METHODS

The study was conducted in the microbiology research laboratory of the faculty of Allied Health Sciences, Kuwait University. Approval of the local ethical committee was obtained.

### Samples

Forty strains of *Klebsiella pneumoniae* were included in this study. These strains were isolated from a variety of clinical specimens submitted to the clinical bacteriology laboratories in Al-Amiri Hospital. They were flagged as ESBL-positive by the Vitek 2 GNI and AST-N020 cards (Bio Merieux, Marcy L'Etoile, France). Samples were grown at 37 °C on Luria Bertani (LB) media (from GIBCO, BRL, Life Technologies, UK), before extracting their DNA.

### Methods for DNA extraction

In the first method, two colonies of overnight growth bacteria were used. The colonies were put in a test tube containing one ml of distilled water and boiled for 10 minutes in a water bath, and then were centrifuged for five minutes at 1000 rpm. Five microliters of the supernatant were used for the PCR. The second method was based on using a National microwave oven (Matsushita Electric Industrial Company, Japan) to heat the bacterial colonies (two colonies dissolved in 500  $\mu$ l distilled water) for 10 seconds, followed by centrifugation for two minutes at 1000 rpm. Similarly, 5  $\mu$ l of the supernatant were used for the PCR.

### Commercial Kits for DNA extraction

Genomic DNA from the same bacterial isolates was extracted for PCR by using Genra Puregene DNA isolation kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. DNA samples were tested by spectrophotometry at dual UV light (260/280) and the ratio was 1.7-1.9 for all samples.

### PCR

PCR was performed on all the DNA samples extracted using the two methods and the commercial kit. Five microliters of the DNA were mixed with 45  $\mu$ l of pre-aliquoted Reddy-Load PCR Mix (from ABgene, UK) containing 1.25 units of Taq DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 mM  $\text{MgCl}_2$ , 0.01% (v/v) Tween 20, 0.2 mM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and 100 pmol of each of the primers indicated in Table 1. The expected sizes of PCR products for the two sets of primers were 308 and 858 base-pairs (bp), respectively (Table 1). For SHV primers, the PCR mixture was incubated for five minutes at 95 °C as an initial denaturation step, followed

**Table 1:** The sequence of primers used in the project to amplify regions of the *blaSHV* and *blaTEM* ESBL genes, as well as the expected sizes of the PCR amplicons

Primer	Sequence	Gene	Expected size of PCR product
SHV-1	5' -CTGGGAAACGGAACTGAATG- 3'	<i>blaSHV</i>	308 bp
SHV-2	5' -GGGGTATCCCGCAGATAAAT- 3'		
TEM-1	5' -ATGAGTATTCAACATTTCGG- 3'	<i>blaTEM</i>	858 bp
TEM-2	5' -CCAATGCTTATTCAGTGAGG- 3'		

by 32 cycles of successive alternating temperatures as follows: denaturation step at 94 °C for one minute, annealing step at 57 °C for one minute, and extension step at 70 °C for one minute. A final extension step at 72 °C for 10 minutes was allowed. On the other hand, and for the TEM primers, the PCR mixture was incubated for five minute at 95 °C as an initial denaturation step, followed by 30 cycles of successive alternating temperatures as follows: denaturation step at 94 °C for 30 seconds, annealing step at 55 °C for one minute, and extension step at 70 °C for one minute. A final extension step at 75 °C for 10 minutes was also allowed. The PCR reaction for both sets of primers was performed in a programmable PCR Thermal Cycler (Perkin Elmer, Wellesley, MA, USA).

#### RFLP

TEM-specific PCR products were digested by *Sau3AI* endonuclease using 10 µl of the PCR product without purification, according to the recommendation of the restriction endonuclease suppliers (Promega, Ltd, UK). The following amounts were used: 5 µl of restriction buffer (10 mM Tris-HCL, pH 7.5, 60 mM NaCl, 7 mM MgCl<sub>2</sub>), 1 µl of BSA (0.1mg/l), 1 µl of restriction enzyme and 4 µl of sterile distilled water. Digestion was carried out for four hours at 37 °C. For SHV, PCR products were digested with 10 U/µl of *NheI* restriction enzyme (Promega, Ltd, UK), 5 µl of restriction buffer (10 mM Tris-HCL, pH 7.5, 60 mM NaCl, 7 mM MgCl<sub>2</sub>), 1 µl of BSA (0.1mg/l), 4 µl of sterile distilled water and 40 µl of the amplified PCR product. Digestion was carried out for a maximum of four hours at 37 °C. Restriction pattern of PCR products for both sets of primers were analyzed by agarose gel electrophoresis, using 2% agarose in 1X Tris acetate EDTA (TAE) buffer, which were then stained with ethidium bromide and visualized by exposure to UV light in a gel documentation system (UVP Company, Upland, CA, USA). A DNA marker from Sigma (Sigma-Aldrich, Inc., Saint Louis, MI, USA) was run on the gel along with the PCR amplicons to identify the sizes of these amplicons.

#### DNA sequencing

DNA sequencing was performed on 10 randomly selected bacterial isolates out of the 40

isolates included in this project as representatives of the whole group PCR products for the SHV gene, obtained from the PCR step above and were taken for sequencing. These products were first cleaned by ethanol precipitation; 25 µl of template suppression reagent (TSR) was added to the pellet, mixed, and finally heated for two minutes at 95 °C. For sequencing PCR, one microliter of each PCR product from the previous step was mixed with 3.2 picomol of either a forward (5'-CTG GGA AAC GGA ACT GAA TG-3') or a reverse primer (5'-GGG GTA TCC CGC AGA TAA AT-3'), and 8 µl of a dye terminator ready sequence reaction mix (Prism™ Ready Reaction Dye-Deoxy™ Terminator Cycle Sequencing Kit, Perkin Elmer, Wellesley, MA, USA). The sequencing PCR reaction was then carried out in the Thermal Cycler programmed to 30 cycles of 96 °C for 20 seconds, 50 °C for 20 second, and 60 °C for four minutes. The products were cleaned again as mentioned above, and the products were kept on ice till the sequencing was run on an automated DNA sequencer (AB13100, Applied Biosystem, Foster City, CA, USA). Sequences results were analysed by the BLAST online search engine (<http://www.ncbi.nih.gov/cgi-bin/BLAST>), with the susceptible strains sequences in the database.

#### RESULTS

PCR amplicons were produced successfully in all DNA samples included in this project. The amplified products obtained with primers specific for both *blaTEM* and *blaSHV* were 858 bp and 308 bp, respectively, which were the expected product sizes of the amplified gene with the set of primers used. That was true whether the DNA was extracted by the two simple methods described here, or using the commercial kit. Figure 1 shows a photograph of agarose gel electrophoresis of these PCR amplicons.

In the RFLP step, *NheI* restriction endonuclease was used to cleave the SHV-specific PCR product, while *Sau3AI* restriction endonuclease was used to cleave the TEM-specific PCR. The results of all the restricted PCR products (SHV or/and TEM) were as expected for each restriction enzyme. The patterns of cutting were similar whether the DNA was extracted by the two methods introduced by the authors, or using the commercial kit (Fig. 2 and 3).



cells, such as boiling or microwave irradiation, was widely used to extract DNA molecules<sup>[5,7,9,10-13]</sup>.

Still, many of the previous methods were either followed or preceded by enzymatic or detergent treatment to obtain DNA for molecular techniques. Many companies have utilized the previous concepts in producing commercial kits that could be used in extracting DNA from a variety of cellular material<sup>[14,19]</sup>. Although providing simpler DNA extraction methods, such kits added extra costs to experiments needing DNA extraction.

In this study, simplified DNA extraction methods to produce bacterial DNA samples were evaluated. The aim was to minimize the time and the need for reagents, while still not affecting the quality of DNA extracted and the productivity of the subsequent molecular techniques. The methods were based on using heat without adding any reagent. Heating bacterial material (suspended in distilled water without any other additions) was achieved by boiling for 10 minutes or microwave irradiation for 10 seconds. It was shown that these two methods have provided enough DNA molecules to perform subsequent molecular biology techniques. The methods were tried on ESBL genes of *Klebsiella pneumoniae*. The ESBL genes were detected by PCR amplification of the DNA sequences coding for *blaTEM* and *blaSHV* ESBL genes. PCR was successful in all cases, giving the expected PCR amplicons. That was additionally verified by performing the same PCR protocol on DNA samples extracted from the same bacterial samples using a commercial DNA extraction kit. PCR amplicons were qualitatively equivalent in all experiments. Furthermore, and in the RFLP experiment, digestion of TEM and SHV PCR products with *Sau3AI* and *NheI* endonucleases, respectively, showed the same fragments and results in all the tested samples whether the DNA used for PCR was extracted by the two methods introduced by the authors or using the commercial kit. Finally, DNA sequencing was also successful in all DNA extraction methods used in this project, giving the expected sequences.

To compare with the work of this study, a limited number of researchers have also used boiling and / or microwave irradiation to extract DNA without any reagents added. However, most of these researchers have boiled their samples or exposed them to microwave irradiation for a time longer than the presented method in this paper<sup>[14-15]</sup> or have subjected their samples to multiple microwave irradiation<sup>[6,15]</sup>. To the best of the authors' knowledge, the report by Merk *et al* was the only one in which the samples were boiled for 10 minutes like in our study<sup>[14]</sup>. Unlike this paper, their samples were blood and lung tissue which were artificially infected with *Burkholderia cepacia*. In addition, their extracted DNA was processed by PCR only. The present paper may

be the first to report using a 10-minute boiling, or as short as 10-second microwave irradiation to extract bacterial DNA suitable to perform three essential molecular biology techniques; namely PCR, RFLP and DNA sequencing.

## CONCLUSION

In conclusion, the presented methods (heat-treatment of bacteria) are very simple, cheap, quick and successful methods for DNA extraction from bacteria in order to be used directly in molecular techniques, yielding excellent results as other more complicated methods for DNA extraction and purification. The findings of this study may probably encourage trying the procedure on other types of biological specimens such as whole blood, culture cells, body fluids and hair.

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