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Analysis of Purity and Concentration *Escherichia coli* DNA by Boiling Method Isolation with Addition of Proteinase-K and RNase

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Abstract

Escherichia coli is a leading cause of Urinary Tract Infections (UTIs) in Indonesia, with approximately 180,000 cases reported annually. The more cases of UTIs, the more PCR diagnosis is needed with an accurate, fast, simple, and economical DNA isolation method. However, currently, there is no DNA purification stage from protein and RNA contaminants in the boiling DNA isolation method. This study aimed to investigate the impact of incorporating Proteinase-K and RNase into the boiling DNA isolation method on the purity and concentration of *E. coli*'s DNA during isolation. The boiling method involved heating to 95°C – 100°C bring to cell lysis and release of cellular components, including DNA. Urine samples were artificially contaminated with *E. coli* at different McFarland standards (0.25, 0.5, and 1). The boiling DNA isolation method was then performed and then analyzed for purity and concentration using a NanoDrop spectrophotometer. This study demonstrated a positive correlation between Proteinase-K and RNase concentrations used in the boiling DNA isolation method and the subsequent increase in DNA purity and concentration. An increase in DNA purity and concentration was obtained even though it was not statistically significant compared to that without Proteinase-K and RNase addition, with *p*-values of 0.245 for DNA purity and 0.353 for DNA concentration. Further research is recommended with higher Proteinase-K and RNase concentrations in the boiling DNA isolation method to achieve improved purity and concentration of *E. coli* DNA. Such enhancements could improve PCR amplification and help diagnose *E. coli*-related UTIs.

Keywords

Boiling Method, DNA purity, DNA concentration, Proteinase-K, RNase.

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INTRODUCTION

Urinary tract infections (UTIs) are frequently linked to *Escherichia coli* infections. *E. coli* is a Gram-negative bacterium that is part of the normal flora in the gastrointestinal tract of humans and animals. However, under certain conditions, it can cause infections in the urinary tract, leading to UTIs. The vast majority, precisely somewhere in the range of 80%, of instances of urinary tract infection are caused by *E. coli* (1). There was a considerable prevalence of UTIs in Indonesia, with an estimated yearly incidence of 180,000 cases (2), as stated in a report published by the Ministry of Health of the Republic of Indonesia in 2014. UTIs have been linked to a significant number of bacteria in the urine. The relevance of bacteriuria is assessed by the presence of pathogenic bacteria in midstream urine at a concentration of $\geq 10^5$ colony-forming units per milliliters (CFU/mL) (3). This concentration is used to measure the number of bacteria in the urine sample.

When *E. coli*, the culture method by using Mac Conkey agar is universally acknowledged as the gold standard, the most reliable and accurate approach (4,5). However, the bacteria detection process by culture methods requires abundant time to gain conclusive results, and this period normally lasts for around two days (6). The presence of low bacterial counts and an increased likelihood of contamination can lead to the reporting of false-negative results (7). Polymerase chain reaction (PCR) techniques were then performed, which have made it possible to detect *Escherichia coli* in a manner that is accurate, specific, and speedy (8,9).

The PCR begins with the DNA isolation. The boiling method is one of the most common techniques for DNA isolation because of its ease of use and potential to produce an adequate number of DNA isolates. The genetic material strands are released from the cell nucleus through physical disruption or temperature changes (10).

E. coli is categorized as a Gram-negative bacterium because its cell walls are relatively thin. Based on the thin cell wall, the bacterium is sensitive to lysis at elevated temperatures resulting in the cellular contents released like DNA (11). The boiling method is a viable choice that should be considered (12).

The employment of spectrophotometric parameters, which quantify the purity and concentration of nucleic acids, particularly DNA and RNA (13), may be utilized to evaluate the quality of DNA isolation. The absorbance ratio calculation at wavelength of 260 nm and 280 nm (λA260/A280) is required for the purity, while absorbance measurements at a wavelength of 260 nm are necessary for the DNA estimation concentration. In most cases, a DNA purity

ratio that falls in the range of 1.8 to 2.0 is generally accepted as "pure" (14). It is possible for the presence of contaminants in DNA to have a detrimental effect on the effectiveness of the Taq Polymerase enzymes, which in turn might result in reduced amplification signals (15).

It has been proven that a viable approach for *E. coli* DNA isolation is through contaminated *E. coli* utilization (16). After the centrifuged process, one may see cellular materials in the urine pellet, including DNA (17). For this reason, the researchers decided to use *E. coli* spiked urine, which is urine samples that were purposefully added with *E. coli* suspension (16). These urine samples were augmented with Units (CFU) of each *E. coli* bacteria suspension obtained from the Optical Density (OD) measurements with a spectrophotometer or densitometer to estimate the concentration of bacteria in suspension (16). The densities of the bacteria ranged from McFarland 0.25 (0.75×10^8 CFU/mL) to McFarland 0.5 (1.5×10^8 CFU/mL) to McFarland 1 (3.0×10^8 CFU/mL) (18).

The DNA purification stages that are designed to remove various biological components from DNA that are not included in the boiling approach. Proteinase-K and RNase are used to remove any protein or RNA contaminants that might be present in DNA samples (19). These enzymes are used in the process of purifying DNA samples. For this investigation, the use of Proteinase-K is warranted because of its capacity to eliminate protein impurities. This elimination is reached through the degradation or digestion of the peptidoglycan layer of the cell wall, which is composed of proteins. Notably, the Proteinase-K effectiveness is not affected by the absence of lysing detergents in the environment. Despite this, it has been discovered that DNA extraction using the boiling approach can still give good results for future analysis, even in the absence of Proteinase-K(20). This was the conclusion reached by the researchers who conducted the study.

The boiling approach does not include a step for the DNA purification to remove RNA impurities from the sample. RNA can absorb UV radiation with a peak wavelength of 260 nanometers, which might be detected as DNA (21). RNases method of action involves the enzymatic breaking of phosphodiester bonds between the 5'-ribose of nucleotides and the phosphate group associated with the 3'-ribose (22). These bonds are formed when a ribose sugar is attached to a phosphate group. The production of 3'-nucleoside phosphate occurs as a direct consequence of this cleavage reaction being followed by hydrolysis (22). The purpose of this research was to investigate whether Proteinase-K and RNase addition to the DNA isolation process using the boiling method may increase the quality

and quantity of the *E. coli* DNA that was obtained.

MATERIALS AND METHODS

Materials

The research employed a quasi-experimental design utilizing a non-equivalent control group design. The study focused on a singular group of urine samples that were contaminated intentionally with *E. coli*. There were nine urine samples used in this study were obtained from healthy individuals. These samples were collected from 3 randomly selected individuals who contributed to multiple urine samples. *E. coli* suspensions from pure cultures or American Type Culture Collection (ATCC) 25922 were added to stimulate bacterial infection.

The concentrations of the bacterial suspensions were standardized using McFarland standards, specifically at levels of 0.25 McFarland (X), 0.5 McFarland (Y), and 1 McFarland (Z), McFarland's variation indicates added to the urine were 0.75 x 10^8 CFU/mL, 1.5 x 10^8 CFU/mL, and 3.0 x 10^8 CFU/mL. The analysis of DNA purity and concentration was conducted using a NanoDrop spectrophotometer.

The samples were tested twice (duplicate) to obtain accurate results. The urine samples were collected from individuals residing in the vicinity of Politeknik Kesehatan Kementerian Kesehatan (Kemenkes) Bandung, specifically from the Faculty of Medical Laboratory Technology located at Jl. Babakan Loa 10A, Cimahi Utara, Cimahi, West Java, Indonesia.

Ethical Statement

The study was carried out at the Bacteriology Laboratory and Molecular Biology Laboratory, part of the Department of Medical Laboratory Technology at Politeknik Kesehatan Kemenkes Bandung. The study received ethical approval from the Health Research Ethics Commission (KEPK) of Politeknik Kesehatan Kemenkes RI Bandung (Certificate no. 26/KEPK/EC/VI/2023).

DNA Isolation Using the Boiling Method

The DNA isolation procedure employed in this study was to use a modified boiling method based on the previous method (10). Each 250 µL urine sample was artificially contaminated with *E. coli* at McFarland standards of 0.25, 0.5, and 1. Subsequently, these samples were transferred using a pipette into individual 1.5 µL microtubes. The samples underwent centrifugation at 12,000 rpm for 10 minutes (Thermo Scientific Heraeus Pico 17 microcentrifuge). Subsequently, the liquid portion, known as the supernatant, was removed, leaving behind the solid mass referred to as the pellet. A volume of 100 µL of 1× Tris-EDTA (TE) buffer was introduced (TE Buffer 1× V6231, Promega). The Proteinase-K (Proteinase-K

solution 4333793, Thermo Scientific, Indonesia) was introduced at predetermined concentrations, and then the tube froze at −80°C for 15 minutes to induce cellular tissue damage. Subsequently, the tube was incubated at 95°C – 100°C for 15 minutes. The solution was allowed to cool to ambient temperature and then supplemented with precise concentrations of RNase (RNase A solution A797C, Promega). The resulting mixture was thoroughly homogenized and incubated at 37°C for 15 minutes. The solution was centrifuged at a speed of 13,000 rpm for 3 minutes. Following centrifugation, the resulting supernatant was carefully transferred into a sterile tube and subsequently stored at a temperature of −20°C. This stored supernatant was used as a DNA template in subsequent experiments.

This study involved the implementation of four distinct treatments. The first treatment, referred to as the control treatment, involved the isolation of DNA using the boiling method. This was done using urine samples that were spiked with *E. coli*, without the inclusion of RNase. The second treatment, denoted as treatment A, also employed the boiling method for DNA isolation. However, in this case, the urine samples spiked with *E. coli* were supplemented with 0.5 µL of Proteinase-K (0.1 mg/mL) and 0.5 µL of RNase (0.02 mg/mL). Similarly, treatment B utilized the boiling method for DNA isolation, but the urine samples spiked with *E. coli* were treated with 1 µL of Proteinase-K (0.2 mg/mL) and 1 µL of RNase (0.04 mg/mL). Lastly, treatment C involved the boiling method for DNA isolation, with the urine samples spiked with *E. coli* being treated with 1.5 µL of Proteinase-K (0.3 mg/mL) and 1.5 µL of RNase (0.06 mg/mL).

The concentrations of Proteinase-K and RNase used in this study were obtained from the calculation according to the addition of stock reagent volume from Promega insert kit TM050 (RNase A) and Thermo Scientific insert kit K0512 (Proteinase-K) and a decrease or increase in the concentration of Proteinase-K and RNase was carried out to determine the significance of improving the quality of the DNA produced.

Statistical Analysis

The primary data utilized in this study was obtained from DNA purity and concentration measurement using a NanoDrop (Lite Spectrophotometer 120V, Thermo Scientific). The data utilized in this study were quantitative nature and analyzed using IBM SPSS Statistics 24 software. The statistical tests employed for analysis included the paired sample t-test and the Wilcoxon test. The data was presented descriptively through the utilization of tables.

RESULTS

The samples used in this research were *E. coli* spiked urine, which is urine samples that had been purposefully added with *E. coli* suspension. The concentrations of bacterial suspensions were standardized using McFarland standards, specifically at levels of 0.25 McFarland (X), 0.5 McFarland (Y), and 1 McFarland (Z). McFarland's variation indicates added to the urine were 0.75 x 10^8 CFU/mL, 1.5 x 10^8 CFU/mL, and 3.0 x 10^8 CFU/mL.

In this research, two obtained data (duplicate) showed almost the same results, and there was no significant difference. The final data on the purity and concentration of DNA is the average of the two data, so the generated data is more accurate. The data obtained from measurements using the NanoDrop spectrophotometer consisted of DNA purity values obtained from the absorbance ratio at wavelengths of 260 nm and 280 nm, as well as DNA concentration values in ng/µL units. The results of DNA purity measurements for *E. coli*-spiked urine samples are presented in Table 1.

Table 1. Results of DNA Purity Measurement in *Escherichia coli*-Spiked Urine Samples

Sample[a]		Treatment[b]			
		Control	A	B	C
Urine 1	X	2.02 ± 0.01	2.02 ± 0.01	1.99 ± 0.03	1.98 ± 0.02
	Y	2.02 ± 0.01	2.03 ± 0.02	1.97 ± 0.02	1.93 ± 0.02
	Z	2.04 ± 0.03	2.02 ± 0.01	1.98 ± 0.02	1.96 ± 0.05
Urine 2	X	1.07 ± 0.02	1.05 ± 0.04	1.06 ± 0.03	1.08 ± 0.01
	Y	1.06 ± 0.03	1.05 ± 0.04	1.07 ± 0.02	1.14 ± 0.05
	Z	1.05 ± 0.04	1.04 ± 0.04	1.17 ± 0.04	1.22 ± 0.03
Urine 3	X	0.97 ± 0.03	0.96 ± 0.02	0.98 ± 0.01	0.97 ± 0.03
	Y	0.98 ± 0.01	0.95 ± 0.04	0.96 ± 0.02	0.98 ± 0.01
	Z	0.97 ± 0.03	0.95 ± 0.04	0.94 ± 0.04	0.99 ± 0.03

[a] Urine spiked *E. coli* with levels of 0.25 McFarland 0.75 x 10^8 CFU/mL (X), 0.5 McFarland 1.5 x 10^8 CFU/mL (Y), and 1 McFarland 3.0 x 10^8 CFU/mL (Z).
[b] Data are expressed as mean ± SD (n = 2)

Table 1 showed that DNA purity gradually increased with higher Proteinase-K and RNase concentration. Increasing DNA purity with higher concentrations of Proteinase-K and RNase could suggest that these enzymes are playing a crucial role in removing unwanted substances. The highest purity of *E. coli* DNA was achieved in treatment C, with the addition of 1.5 µL of Proteinase-K (0.3 mg/mL) and 1.5 µL of RNase (0.06 mg/mL) to the spiked urine samples during the boiling DNA isolation method. The DNA concentration measurements for *E. coli*-spiked urine samples are presented in Tables 2.

Table 2. Results of DNA Concentration Measurement in *Escherichia coli*-Spiked Urine Samples

Sample[a]		Treatment[b]			
		Control	A	B	C
Urine 1	X	518.0 ± 8.77	519.2 ± 7.35	620.6 ± 11.45	637.8 ± 3.11
	Y	408.4 ± 11.74	402.9 ± 8.63	628.4 ± 10.04	735.0 ± 5.80
	Z	420.2 ± 17.11	480.9 ± 6.22	796.8 ± 13.15	964.5 ± 5.09
Urine 2	X	219.2 ± 5.23	242.9 ± 11.45	287.7 ± 15.83	185.0 ± 12.87
	Y	281.2 ± 5.94	297.8 ± 15.98	279.6 ± 14.14	286.6 ± 9.19
	Z	322.4 ± 15.70	299.5 ± 14.99	281.4 ± 19.94	423.3 ± 11.03
Urine 3	X	450.5 ± 21.50	497.2 ± 3.11	485.2 ± 3.11	520.6 ± 14.28
	Y	339.6 ± 14.28	591.7 ± 8.20	518.6 ± 7.91	512.6 ± 4.10
	Z	430.5 ± 10.32	589.2 ± 11.88	626.2 ± 7.35	721.9 ± 5.23

[a] Urine spiked *E. coli* with levels of 0.25 McFarland 0.75 x 10^8 CFU/mL (X), 0.5 McFarland 1.5 x 10^8 CFU/mL (Y), and 1 McFarland 3.0 x 10^8 CFU/mL (Z)
[b] Data are expressed as mean ± SD (n = 2)

Table 2 revealed that DNA concentration increased as the Proteinase-K and RNase concentrations were elevated. The highest DNA concentration of *E. coli* was obtained from the boiling DNA isolation method with the addition of 1.5 µL of Proteinase-K (0.3 mg/mL) and 1.5 µL of RNase (0.06 mg/mL) to the spiked urine samples. This suggests that the enzymatic activities of Proteinase-K and RNase might have facilitated the release and purification of DNA from the sample, resulting in a higher yield of DNA. The descriptive analysis of the average values for DNA purity and concentration for each treatment on urine samples is presented in Table 3.

Table 3. Average Values of DNA Purity and Concentration Based on Variation in – Urine Samples

Sample	Treatment	DNA Purity Ratio Values (A260/A230)	DNA Concentration (ng/µL)
Urine 1	Control treatment	2.02	448.8
	Treatment A	2.02	467.6
	Treatment B	1.98	681.9
	Treatment C	**1.95**	**779.1**
Urine 2	Control treatment	1.06	274.2
	Treatment A	1.04	280.0
	Treatment B	1.10	282.9
	Treatment C	**1.14**	**406.8**
Urine 3	Control treatment	0.97	406.8
	Treatment A	0.95	505.6
	Treatment B	0.96	543.3
	Treatment C	**0.98**	**585.0**

Remark: Bold mark indicates the highest DNA purity and concentration value was obtained from treatment C

Table 3 showed that the average value of purity and concentration of DNA for each treatment was increased. The highest DNA purity and concentration were obtained from treatment C, in which the purity and concentration of DNA is considered the best compared to treatment A and B. Treatment C is the boiling method of DNA isolation with the addition of 1.5 µL of Proteinase-K (0.3 mg/mL) and 1.5 µL of RNase (0.06 mg/mL).

A statistical test was conducted to assess the impact of increased DNA purity and concentration. The average *p*-values for DNA purity in each sample were 0.245. Similarly, the *p*-value for DNA concentration was 0,353. It indicates there was no significant impact between the control and Treatment A, B, and C, considering various variations in Proteinase-K and RNase concentrations added to the boiling DNA isolation method. There was also no significant influence between the control and Treatment A, B, and C, considering various variations in Proteinase-K and RNase concentrations added to the Boiling DNA isolation method.

DISCUSSION

The levels of DNA purity in sample urine 1 ranged from 1.93 to 2.04. In urine sample number 2, the values for DNA purity ranged from 1.04 to 1.22, whereas in urine sample number 3, the values for DNA purity were in the range of 0.94 to 0.99. Normal urine samples for this study were obtained from various participants, which may account for some of the observed differences. This study sample is more representative because each urine has different contents, such as protein levels and urine-borne bacteria. In DNA extraction the purity level of DNA is strongly influenced by protein and RNA contaminants. However, there are limitations to this research. This research did not check the protein urine level or bacteria other than *E. coli* in the urine before DNA isolation.

Urine sample 1 received the control procedure yielded an average DNA purity of more than 2.0. However, there was a discernible improvement in the DNA's purity after RNase addition. This impact might be the result of the presence of bacterial RNA in urine sample 1, which the NanoDrop spectrophotometer may have misinterpreted as DNA during the measurement because the control treatment did not contain ribonuclease (RNase). It might be explained by the absence of RNase (23).

The average values for DNA purity in urine samples 2 and 3 were between 1.06-1.14 for urine sample 2 and between 0.95-0.98 for urine sample 3. These results are below the range of pure DNA (1.8-2.0). The low purity may be due to high protein contaminants in the samples. These protein contaminants may have originated from non-lysed cell components or bacterial proteins. Alternatively, these protein contaminants

may have been present naturally. These protein pollutants can be found at a wavelength of less than 280 nm (24). Insufficient Proteinase-K may explain the persistently high protein contaminant levels in urine samples 2 and 3.

During the boiling technique, no phase that involved washing the DNA with 70% ethanol, which contributes to decreased DNA purity (25). The ethanol addition is necessary to dissolve the soluble residual pollutants. However, DNA, which is insoluble in ethanol, will precipitate in the bottom of the microtube because it cannot be dissolved in ethanol.

The increase in DNA purity was negligible, at about 0.01, regardless of the treatment. It is most likely because of the differences in the small quantities of administered Proteinase-K and RNase. On the other hand, the administration of Proteinase-K tends to improve the purity of the DNA. Proteinase-K, even in minute doses, can halt the process of protein breakdown. When the concentration of Proteinase-K is insufficient, the available amount of enzyme for the cleavage of proteins is restricted. As a direct consequence, the protein breakdown process is slow or fails.

The addition of Proteinase-K as well as RNase resulted in an increase in the DNA amount in the sample. Proteinase-K is thought to be responsible for this phenomenon because it lyses cells, removes protein impurities and plays a function in the breaking down process of protein pollutants. Proteinase-K is an enzyme that helps in the process of cell lysis by cleaving the carboxyl amino groups in the peptide bonds that are present in the peptidoglycan layer of the cell wall. It allows the cell to be more easily destroyed. Therefore, Treatment C, which had the highest levels of both additional Proteinase-K and RNase, produced the best results in terms of DNA concentration (26).

In this investigation, the bacterial suspension that was put into the urine underwent varying turbidity processes at levels of 0.25, 0.5, and 1 McFarland units. This change reflected the bacteria number that had been added to the urine, and it corresponded to roughly 0.75 x 10^8 CFU/mL, 1.5 x 10^8 CFU/mL, and 3.0 x 10^8 CFU/mL, respectively. The rise in the DNA content in urine samples that had been added with *Escherichia coli* was precisely equal to the increase in bacterial cell numbers in those samples. The relationship occurs because *Escherichia coli* is a prokaryotic cell, which contains a single chromosome that has DNA. Therefore, the amount of recovered DNA increased in proportion to the number of *Escherichia coli* bacteria that were present (27).

Based on the data that were acquired, it is clear that the rise in DNA concentration for each sample varies unpredictably. These

differences in results could be attributed to technical factors that occurred during the measurement process. These technical factors include an incomplete homogenization before measurement using the spectrophotometer, the possible adherence of some DNA to the microtube, and pipetting errors, all of which could lead to lower recorded DNA concentrations in the spectrophotometer results.

There were no discernible differences between the control treatment and the treatments that utilized varied amounts of Proteinase-K and RNase that were investigated in this study, as evidenced by the fact that the values for DNA purity and concentration that were obtained each had a *p*-value that was >0.05.

CONCLUSIONS

The DNA purity and concentration exhibited a trend of improvement with the addition of varying Proteinase-K and RNase concentrations addition in the boiling DNA isolation method. However, the increase in DNA purity and concentration was not statistically significant. Therefore, further investigation with higher Proteinase-K and RNase concentrations in the boiling DNA isolation method is needed to achieve improved DNA purity and concentration in *Escherichia coli*.

AUTHOR CONTRIBUTIONS

All of author conceived and designed the analysis, performed the analysis, collected the data and wrote the paper.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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