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RESEARCH ARTICLE

RNA extraction from urine sediment: A cost-effective protocol for gene expression analysis in renal pathology.

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Abstract: Urine is an appropriate choice of specimen to study the biomarkers for metabolic and renal disorders because it is readily available with less harm to the patients. However, RNA extraction from voided urine is challenging due to the presence of RNases and cell scarcity. This study aims to optimize a protocol for RNA extraction from urine samples for gene expression studies in renal pathology. Hundred and two urine samples were collected from both healthy controls (HC) (n = 15; 54 ± 11 years) and chronic kidney disease (CKD) patients (n = 87; 56 ± 10 years) and centrifuged at 6,500 g for 20 min at 4 °C to obtain sediment. RNA was extracted from urine sediments using a phenol-based technique. The extracted RNA was quantified and reverse-transcribed into complementary DNA (cDNA). Reverse transcriptase quantitative polymerase chain reactions (RT- qPCR) were carried out using 2 ng of template cDNA to amplify the housekeeping gene, β2- microglobulin (*B2M*). The total yield of RNA from CKD and HC samples were 718 ± 164 ng and 790 ± 231 ng, respectively, and a statistically significant difference was not observed between the two study groups ($p > 0.05$). The urinary RNA recovery was significantly increased with CKD progression ($p < 0.05$). Further, the results show that urine volume, gender, and serum creatinine level significantly influence the RNA yield in only disease groups ($p < 0.05$). The mean threshold cycle (Ct) values for *B2M* amplification of CKD and HC were 27.36 ± 3.09 and 20.97 ± 3.90, respectively. This modified phenol-chloroform-based urinary RNA extraction method is less expensive and does not require pre- and post-purification steps. It provides a higher yield of RNA with less inhibition to qPCR and is sufficient for downstream applications than column-based techniques.

Keywords: Chronic kidney disease; cDNA synthesis; Gene expression; RNA extraction; RT-qPCR.

INTRODUCTION

RNA is a macromolecule that regulates the functions of all living cells and tissues through gene expression. Unlike DNA molecules that convey genetic information from genomic sequences, the study of RNA offers valuable information regarding the metabolic status of an organism. (Manning & Cooper, 2017). Determining the physiological or pathological state of an organism is almost always related to gene expression. (Sharp *et al*., 2022). Emerging with advanced molecular techniques, proteomic and transcriptomic studies using non-invasive methods are of utmost importance for disease prognosis and diagnosis (Petra *et al*., 2022).

Biological samples like whole blood, serum or plasma, tissues, and biopsy samples are routinely used in CKD diagnosis. However, urine is the best choice of specimen for non-invasive disease prognosis and diagnosis, especially for renal pathology (Latt *et al.*, 2022), metabolic, and other systemic disorders (Neisius *et al*., 2016). Cellular and cell-free components in urine are derived from the upper or lower urinary system, including the kidney, ureter, bladder, prostate, and urethra, providing information related to urological conditions. Tissue transcriptome-derived urinary biomarker identification reduces the risk of obtaining renal biopsy (Ju *et al*., 2015). In addition, glomerular filtrate contains an abundance of transcripts and has more relaible information related to systemic and metabolic diseases (Bazzell *et al.,* 2018). Unlike other liquid biopsies, RNA isolation from voided urine is still challenging, mainly due to ribonuclease (RNase) enzymes in the environment and urine, which degrade RNA. In addition, the acidic pH of urine, the presence of bacteria in urine, either due to infection or following the contamination during sample collection, and prolonged storage of urine inside the bladder could degrade RNA before sample processing (Mengual & Olivan, 2018).

Previous studies reported different methodologies for RNA isolation from biological specimens: 1. One-step acid guanidinium thiocyanate-phenol-chloroform (GTPC) method using phase separation technique; 2. Silica-based spin column technologies; 3. Magnetic beads-based technologies (Chomczynski & Sacchi, 2006; Tavares *et al*., 2011). The acid-based GTPC RNA extraction method is a conventional and gold standard technique used for RNA extraction, irrespective of the type of samples. GTPC technique uses guanidinium thiocyanate, a chaotropic agent that lyses the cells and denatures proteins and RNase enzymes. The water-saturated phenol separates RNA and DNA during phase separation in acidic pH, and ice-cold isopropanol precipitates RNA (Chomczynski and Sacchi, 2006). The major advantage of this technique is comparably

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low cost compared to other methods and is also suitable for low-yield samples. (Farrell JR, 2009). However, the protocol must be optimized for different types of samples before routine use.

The accuracy of gene expression analysis mainly depends on the quantity and quality of the RNA used for the experiment. Studies would produce results that do not reflect the patient's condition unless the sample submitted for the study is pure. Precautions should be maintained to avoid RNase contamination from sample collection to cDNA synthesis since RNases are freely present everywhere. Precautions should be taken to preserve the integrity of RNA in every step of the procedure, including sample collection, transportation, storage, and cDNA synthesis. Several RNA preservation methods, such as the use of chemical preservatives like RNA later, Norgen preservatives, and buffers containing EDTA and freezing at -80 °C would maintain the integrity of RNA (Camacho-Sanchez *et al*., 2013). The best way to maximize RNA integrity is by "working fast and working cold" (Farrell Jr, 2009).

Further, studies reporting RNA extraction from urine samples require pre-and post-extraction processing steps like concentration and purification. Therefore, it needs additional time and cost to yield urinary RNA for gene expression studies. Thus, this study optimized a total RNA extraction protocol from urine sediment with minimal reagent cost and procession steps and studied the effect of other pathophysiological factors on the RNA yield. The developed protocol uses the fundamental approach of the acid guanidinium thiocyanate-phenol-chloroform method with modification to yield a pure and high amount of RNA for downstream applications (Chomczynski and Sacchi, 2006). This optimized technique was validated with the RT-qPCR technique using a housekeeping gene and proved to be used for gene expression analysis in clinical settings.

MATERIALS AND METHODS

Ethical clearance

Ethical clearance was obtained from the Committee for Ethical Clearance (CEC) of the Postgraduate Institute of Science, University of Peradeniya, Sri Lanka (Reference No: CEC_PGIS_2020_08). Before the sample collection, informed written consent was obtained from all the study participants.

Sample collection and processing

A cohort of chronic kidney disease (CKD) patients (n = 87) attending the nephrology clinic, General Hospital in Vavuniya and healthy volunteers (n = 15) residing in the Kandy District without any symptoms of any diseases and did not have past medical history of any chronic illnesses were recruited for the study during the period of November 2020 to March 2022.

The second-morning urine samples were collected from all the study groups. The "clean catch mid-stream urine sample collection" technique was used in this study. All urine samples were collected into RNase- free 100 mL urine collection containers kept on gel ice packs immediately after collection and transported to the National Institute of Fundamental Studies (NIFS), Kandy. About 10 – 90 mL urine sample (depending on the output of CKD patients) was collected from each study participant. Around 1.5 mL of urine per sample was aliquoted into sterile microcentrifuge tubes and stored at 2 – 8 °C to be used for culturing. The remaining urine samples were stored immediately at -80 °C until RNA extraction.

RNA extraction

Reagents were prepared using diethylpyrocarbonate (DEPC) treated autoclaved water according to the protocol developed by Chomczynski and Sacchi (2006). The RNase-free environment was maintained throughout the work.

Urine samples were transferred into either 15 mL or 50 mL conical centrifuged tubes (based on the volume) and centrifuged at 6,500 g for 20 min at 4 °C using a refrigerated centrifuge (Eppendorf® 5,430 centrifuge, Germany). 100 – 150 µL of cell pellet was resuspended with 500 µL of lysis buffer containing guanidinium thiocyanate. The vial was mixed well and vortexed for 15 s to lyse the cells completely. The mixture was incubated on ice for 5 min. A 100 µL of 3 M Sodium acetate and 500 µL water-saturated phenol (pH 4.0) was added to the mixture and was mixed well by inverting. Then 200 µL of freshly prepared Chloroform: Isoamyl alcohol (49:1) was added to the mixture and vortexed for 15 s and was centrifuged at 14,000 g, 4 °C for 15 min (Hermle Z326, microcentrifuge, USA). The aqueous phase was carefully transferred into another microcentrifuge tube, and an equal volume of ice-cold isopropanol was added. The vial was incubated at -20 °C for 1 h. Then microcentrifuge tube was centrifuged at 14,000 g, 4 °C for 30 min. The supernatant was discarded, and the pellet was washed with 75% ethanol twice and centrifuged at 14,000 g, 4 ˚C for 8 min. The supernatant was discarded without disturbing the pellet. The vial was kept in an inverted position under direct airflow for 5-10 minutes to dry the pellet. Then the gel-like pellet was dissolved in 12 µL of nuclease-free water. The extracted RNA was stored immediately at -80 °C until it was used for cDNA synthesis.

RNA quantification and integrity checking

RNA was quantified using Quantifluor ST fluorometer (Promega, USA) using Quantifluor ™ RNA System according to the manufacturer guidelines. One percent native agarose gel electrophoresis using 1 X TAE buffer was used to check the integrity of the extracted RNA.

Complementary DNA (cDNA) synthesis

Reverse transcription was carried out using the GoScript ™ Reverse Transcription kit (Promega **Cat**.# A5000). The reverse transcription reaction mixture and template RNA reaction mixture were prepared separately using the components provided with the kit according to the manufacturer protocol with some modifications. Briefly, the template RNA reaction mixture was prepared using 50 – 200 ng of template RNA, 1 µL of random primers, and 1 µL of oligo- dT. The reaction mixture was kept on the heating block at 70 °C (thermal cycler) for 5 min and

immediately cooled on an ice bath at 4 °C for 5 min. Then reverse transcription mixture consisted 7.8 µL of nuclease-free water, 4 µL of 5X buffer, 1.2 µL of 25 mM $MgCl_2$, 1 µL of PCR nucleotide mix, and 1 µL of reverse transcriptase was added to the template RNA reaction mixture. Finally, a 20 µL volume of cDNA was prepared with the primer annealing at 42 °C 60 min and inactivation of RT enzyme at 70 °C for 15 min using a thermal cycler. Synthesized cDNA was stored at -20 °C until it is used for the qPCR.

RT-qPCR

Synthesized cDNA was subjected to qPCR amplification using the SYBR Green I (Invitrogen) marker dye for fluorescent real-time detection using the Rotor Gene-Q PCR machine (Qiagen). Briefly, the PCR master mixture consisted of 4 µL of 5X buffer, 0.5 µL of forward and reverse primers (10 µM each), 0.8 µL of 25 mM $MgCl_2$, 2 µL of 1mM dNTPs, 1 µL of 2X SYBR green, 0.125 µL of 5U/µL Taq DNA polymerase (Promega), 12.075 µL of DNase-free water and 4 µL of cDNA (2 ng) to make a final reaction mixture of 25 µL. Forward and reverse primers of the housekeeping gene, β-2 Microglobulin (*B2M*) (Accession No: NM 004048.2) selected from the literature (Koop *et al.,* 2003) were F: 5'TGCCGTGTGAACCAT GTGA- 3' and R: 5'CCAAATGCGGCATCTTCAA-3' respectively. PCR conditions were optimized as initial denaturation at 94 °C 5 min, and cycling conditions as denaturation: 94 °C for 30 s, annealing: 60 °C for 30 s, and extension: 72 °C for 30 s. The Rotor-Gene 2.0.3.2 software (Qiagen) was used to obtain the threshold cycle (Ct) values. The specificity of all individual amplification reactions was confirmed by melt curve analysis.

An inhibition plot was carried out with a serially diluted cDNA: 80 ng, 40 ng, 20 ng, 10 ng, 2.5 ng, and 0.625 ng to show the inhibition rate and study the efficiency of the qPCR reaction.

Urine culture

All the urine samples were cultured using urine-specific culture media: Cystine lactose electrolyte-deficient agar (CLED). Briefly, 1 µL of uncentrifuged urine samples were inoculated into the CLED agar plates and incubated overnight at 37 °C. The results were used to study the effect of microbes on RNA recovery.

Statistical analysis

Descriptive statistics were used to calculate the mean and standard error of the mean for RNA yield. Two-sample t-test and one-way ANOVA were used to study the effect of influencing factors on RNA yield. A correlation study was carried out using regression analysis. p-values < 0.05 were considered as significant.

RESULTS

Study population

Hundred and two study participants were employed in this study; 59 males and 43 females. Characteristics of the study groups are summarized in **Table 1**. The mean age of study groups was 56 ± 10 years and 54 ±11 years in CKD and Healthy controls (HC), respectively. HC was selected based on devoid of current and history of any illness. They were screened using dipstick urine analysis before being considered in this study. CKD patients were recruited as sub-category based on the aetiology of CKD including CKD with diabetes mellitus (n = 20), CKD with hypertension (n = 37), CKD with both diabetes and hypertension (n = 11) and CKD derived from other etiology (n = 19).

The mean ± SD serum creatinine level of early (n = 37) and late-stage CKD (n = 50) were 1.63 ± 0.36 mg dL^{-1} and 4.45 ± 2.61 mg dL^{-1}, respectively. The mean urine volume obtained from CKD was 51.32 ± 21.68 mL, and HC was 81.67 ± 10.80 mL.

The yield of total RNA from urine sediment

The urinary total RNA yield of healthy and diseased patients was studied. The results showed that the yield of total RNA was 718 ± 164 ng in CKD and 790 ± 231 ng in HC. The total yield of RNA isolated from female and male study groups was 1159 ± 275.26 ng and 414 ± 133.70 ng, respectively. An indication that the mean RNA yield was 35.72 % higher in females than males (**Figure 1: C**) There was no statistically significant variation in the RNA yield between the two study groups (CKD and HC) (p > 0.05).

Among the CKD study group, the RNA recovery from urine samples was 714 ± 390 ng in diabetic CKD and 742 ± 234 ng in hypertensive CKD. For the patients who have diabetes as well as hypertension, the value was 837 ± 579 ng, and it was 602 ± 308 ng for other CKD aetiologies. Further analysis was carried out to find the difference in RNA yield with disease progression, and the results showed that total RNA yield was 534 ± 214 ng in the early- stage (stage 1–3) and 854 ± 237 ng in late- stage (stage 4–5) of the disease condition. The findings show a significant increase in urinary RNA recovery with disease progression (p < 0.05). Further, **Figure 2** represents the agarose gel electrophoresis pattern of the extracted RNA

Table 1: Characteristics of the study groups (n = 102)

	Age (Years)	Gender		Chronic diseases other than CKD				sCr (mg dL^{-1})
		Male	Female	HT	DM	HT + DM	other	
CKD (n = 87)	56 ± 10	53	34	37	20	11	19	3.25 ± 0.26
HC (n = 15)	54 ±11	6	9	-	-	-	-	-

HT: Hypertension, DM: Diabetes Mellitus, sCr: Serum Creatinine, HC: Healthy Control

from the GTPC method.

The effect of physiological and pathological factors such as age, gender, the volume of urine, the stage of CKD, and the possible contaminations influence the yield of RNA were analyzed separately and summarized in **Figure 1** and **Table 2**, respectively. This study revealed that the above factors did not affect the RNA yield in healthy controls ($p > 0.05$). However, a significant influence on the RNA yield ($p < 0.05$) of the disease group was observed for urine volume, gender and serum creatinine level. The urine culture results showed that out of 87 CKD patients, 26 were culture positive, and the yield of RNA was 995 ± 420 ng, which was higher than patients with culture-negative (599 ± 151 ng).

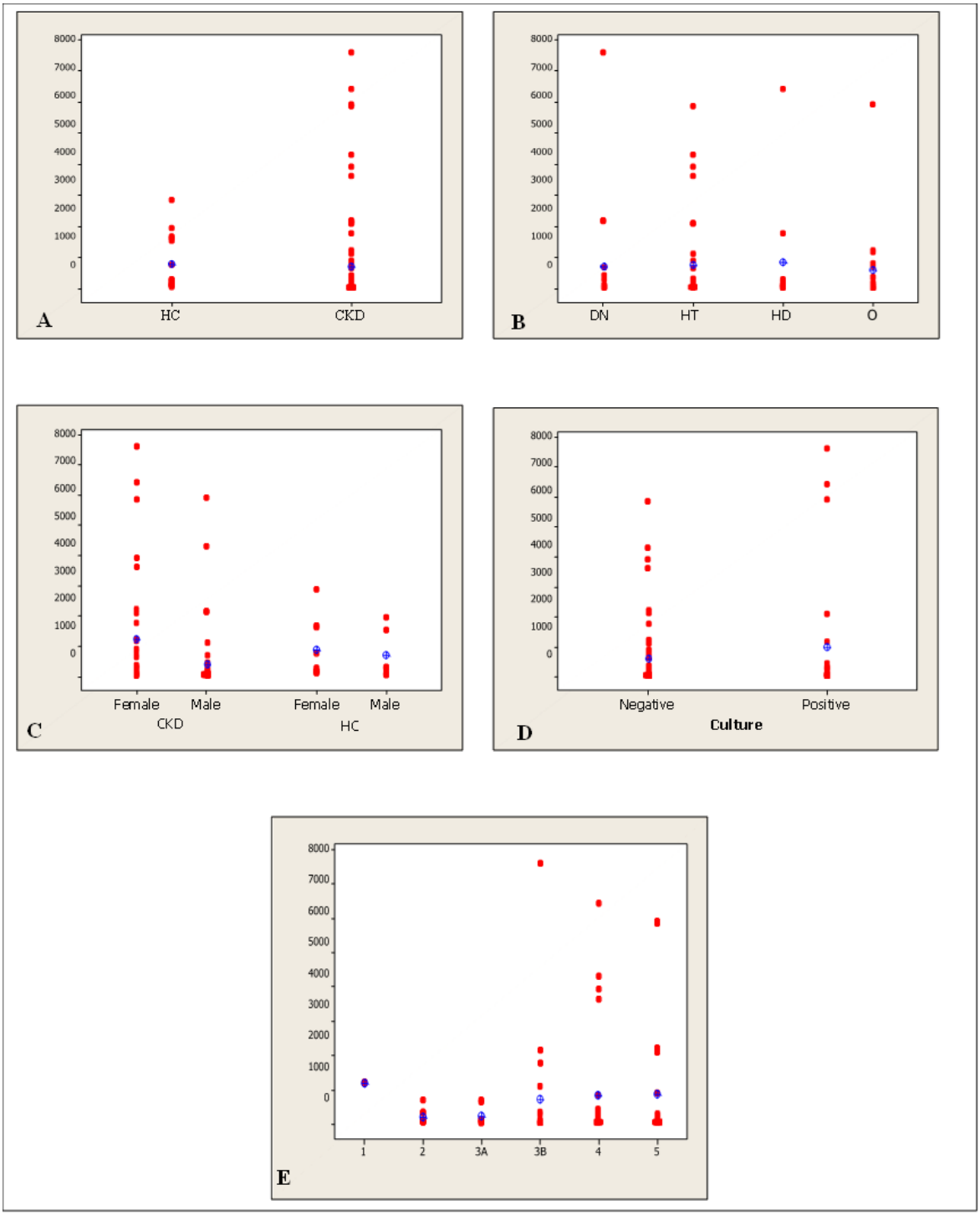

Figure 1: Interval plot of Total RNA yield between two study groups: Healthy Control (HC) and CKD (A), different study groups of CKD, such as Diabetic nephropathy (DN), Hypertension (HT), Both hypertension and diabetes (HD) and Other causes of CKD (O) study group (B), gender variation among two study group (C), the effect of the presence of bacteria (D) and CKD stage(E).

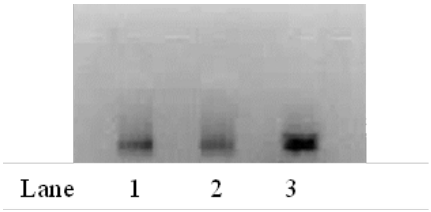

Figure 2: Agarose gel electrophoretic profile of total urinary RNA for study groups. RNA concentration, Lane 1: 69.05 ng/µL; Lane 2: 75.44 ng/µL; Lane 3: 326.50 ng/µL.

RT-qPCR validation

The inhibition plot of RT-qPCR showed that the R^2 value was 0.996, indicating an acceptable fit and slope of -3.94, which gave PCR efficiency of 0.80. Amplification was obtained for the *B2M* gene even at a very minute cDNA concentration of 0.625 ng with a low threshold cycle (Ct) value: 25. The standard curve of the *B2M* gene obtained from healthy groups in **Figure 3** showed an acceptable Ct value (Ct =~26) for low input cDNA template (0.625 ng). It indicates that this method is suitable for even low yield of RNA obtained with this protocol with minimum inhibition for PCR reaction.

Table 2: Factors influencing the yield of total RNA from urine sediments

Factors	Study groups	Significance level (*p*-value)
Gender	HC	0.737
	CKD	0.010‡
Age	HC	0.847
	CKD	0.995
Urine volume	HC	0.788
	CKD	0.000‡‡
Serum creatinine	HC	-
	CKD	0.005‡‡
Presence of bacteria/contaminations	HC	-
	CKD	0.271
Causes of CKD	HC	
	CKD	0.0981

One-way ANOVA was used to calculate the significance level. HC: Healthy controls; CKD: Chronic kidney disease; ‡Significance at the level of 0.05 and ‡‡Significance at the level of 0.01.

The mean Ct value of the *B2M* gene amplified in each study group was analyzed separately and shown in the box plot (**Figure 4**). The mean ± SD Ct value of CKD and HC was 27.36 ± 3.09 and 20.97 ± 3.90, respectively. The specificity of each qPCR reaction was confirmed using melt curve analysis. **Figure 5** shows that the melting temperature of the amplified PCR product was between 83.5 to 84.2 °C. The melting curve showed no unintended product amplification and primer dimer formation in qPCR reactions.

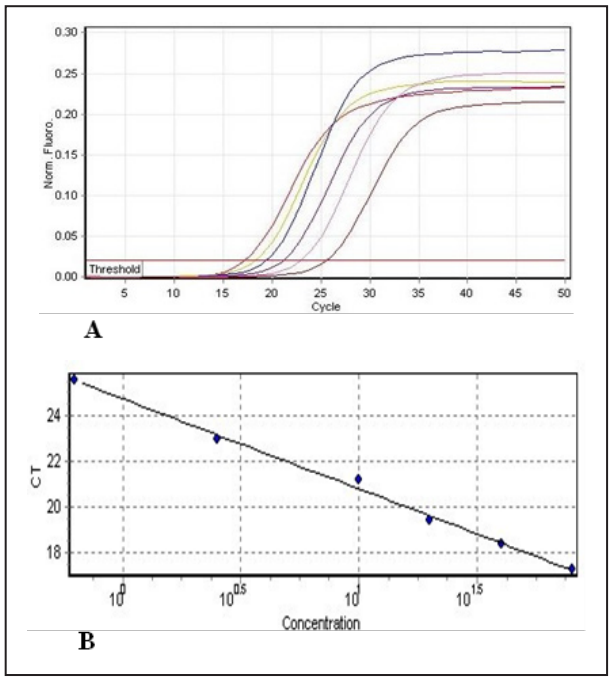

Figure 3: qPCR amplification curve of *B2M* gene and Ct values (A) for serially diluted cDNA (from 80 ng to 0.625 ng) and the standard curve (R^2 = 0.996) (B).

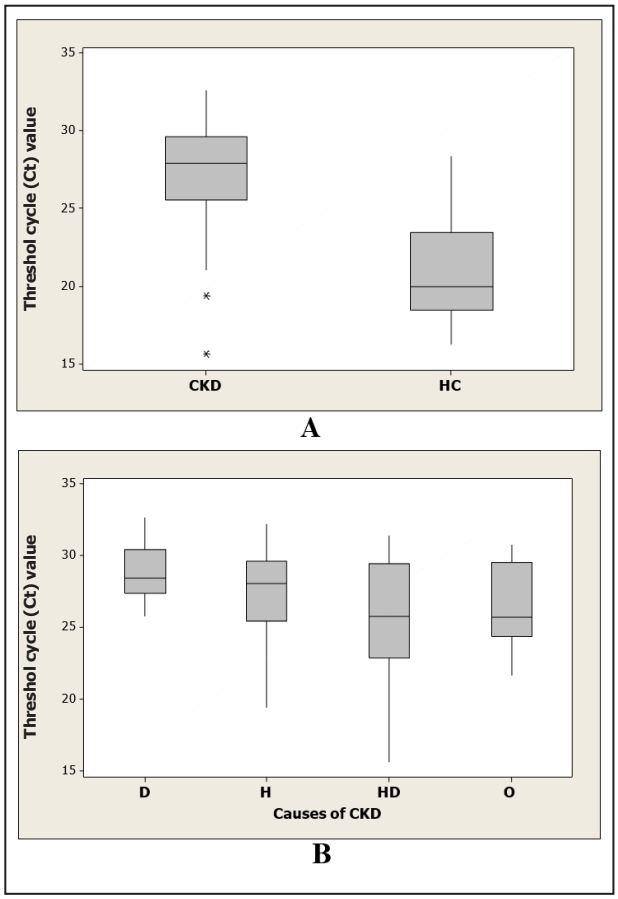

Figure 4: Box plots showing median values and range of threshold cycle (Ct) values of *B2M* gene studied in two groups: CKD and Healthy Control (HC) (A); and sub-category of CKD study groups (B). Outlier is depicted by (*) for values exceeding 150% IQR.

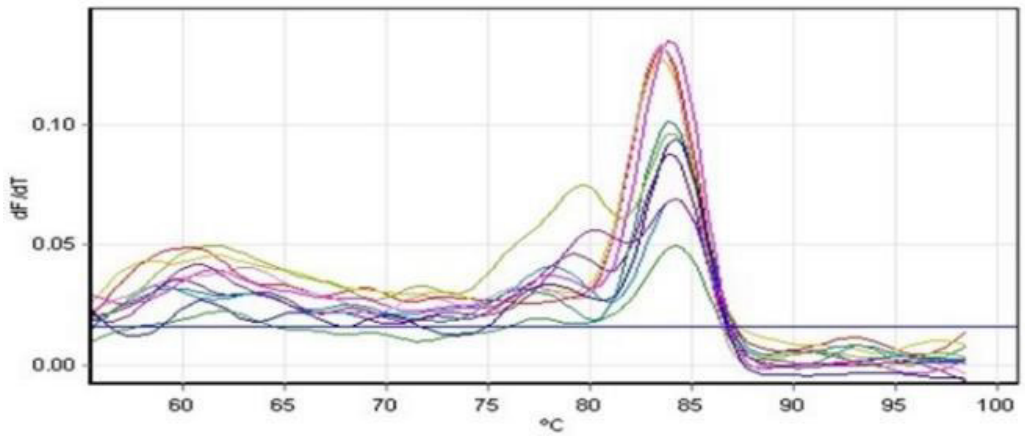

Figure 5: Melt curve analysis of qPCR product of *B2M* gene amplification

DISCUSSION

Researchers have focused on developing simple, cost-effective methods to isolate RNA from various biological samples for decades. In this study, a protocol was optimized to extract total urinary RNA from centrifuged urine sediment using a cost-effective modified phenol-chloroform extraction method. The techniques was validated with RT-qPCR using the housekeeping gene, *B2M*. This current phenol-based RNA extraction technique is comparably less expensive than either silica or magnetic beads-based methods. The silica-based spin column technique, widely incorporated with commercial kits, is easy to perform and less time-consuming than acid-based phenol-chloroform extraction techniques (Bradley *et al*., 2019). However, these techniques are still costly (Escobar and Hunt, 2017). The suitability of these techniques for research-based studies is questionable since these yields relatively low RNA concentrations, especially for samples containing a low amount of RNA (Bradley *et al*., 2019).

Magnetic beads-based methods are relatively simple and use magnetic particles incorporated with ligands to bind RNA during extraction. The primary benefit of this method is that it eliminates the use of a centrifuge (Ali *et al*., 2017) meanwhile, it inhibits PCR reactions and is unsuitable for gene expression studies (Franzreb *et al*., 2006). Further, these rapid methods are still costly and could not be affordable for a large batch of samples, especially for low-income- based countries like Sri Lanka (Toni *et al*., 2018).

In addition to the cost-effectiveness, the current study proved that the recovery of total RNA from the present protocol is enough and suitable for gene expression studies, especially for samples yielding a relatively low amount of RNA.

Bradley *et al*. conducted a study in 2019 to compare three different urine RNA extraction protocol in a clinical setting. Two commercial urinary RNA isolation kits (ZR urine RNA isolation kits and RNA nano prep kits) and phenol-based TRI reagent protocols were used. The results revealed that the phenol-based TRI reagent RNA extraction method yielded more RNA (median yield = 843 ng) than other kits-based methods. In support of the above literature findings, the present study also had more RNA yield (n = 102; 728 ± 143 ng) using the phenol-based method.

Another study by Monteiro *et al.* in 2016 also supports the present finding. The study revealed that the RNeasy mini kits use rapid column-based techniques that yield relatively low RNA concentrations for urine samples (n = 4; 21 ± 6.9 ng/µL) and are not enough for downstream analysis. Therefore, they used another protocol (modified phenol-based method) to extract total RNA from urine samples. In the above modified phenol-based method, they used glycogen and a combination of glycogen and sodium acetate (pH = 5.2) to enhance the precipitation of RNA during the isopropanol precipitation step. However, our current protocol yielded more RNA from urine sediment without using any RNA carrier molecules like glycogen. Monteiro *et al.* (2016) demonstrated that even though the use of glycogen resulted in more RNA yield, which requires additional purification steps. The purification using RNA clean-up kits is needed since glycogen inhibits downstream application. Therefore, this is not a cost-effective method for RNA extraction (Monteiro *et al*., 2016).

Further to the RNA yield, the suitability of recovered RNA for gene expression studies almost always depends on the integrity and purity of RNA. Another comparison study was done to check the suitability of RNA extracted by various techniques and qPCR amplification of the housekeeping gene. *GAPDH* gene expression was not detected in more than 80% of samples extracted using the spin column technique. The above unacceptable results might be due to the extensive degradation of RNA in the column-based method. Meanwhile, Ct values ranging from 22.99 to 30.06 resulted for all samples extracted using the tri-reagent protocol. Therefore, the suitability of the silica-based column technique to extract total urine RNA for gene expression is still questionable (Yazdani *et al*., 2019). However, the present protocol suggests that it could be a better tool for gene expression study from urine samples as all the extracted RNA (100%) was detected using the *B2M* gene with good Ct value, ranging from 15.96 to 32.67.

Further, analysis was carried out to demonstrate the variation of the RNA yield and the influencing factors such as gender, age, urine volume, disease progression, and the presence of bacteria. Statistical data of the current study

confirmed that the RNA recovery was not influenced by gender, especially in control groups. However, in contrast, the female study groups among CKD patients yielded a relatively high amount of RNA than males ($p < 0.05$). A recent study proved that it is common for females to excrete squamous epithelial cells with urine which could be a source of more RNA from cellular elements (Chen *et al*., 2022).

The present study proved that RNA yield was higher in the late stage of CKD than early stage and showed a positive correlation with serum creatinine level in CKD patients ($r^2 = 85.59$). Urinary excretion of cellular contents resulting from damaged renal tubular cells is increased along with the advancement of CKD. Therefore, this research finding revealed that the RNA recovery from urine increases with the disease progression and could be an insight into novel urinary biomarker identification in CKD. Further studies are required to correlate cellular type and its content and the RNA yield with disease progression (Shukuya *et al*., 2016).

Urine volume is another critical factor for gene expression studies, especially for renal disorders, because urine output decreases with the advancement of CKD. Unlike the blood and tissue specimen, the volume of urine samples and their constituents (cells) obtained from each subject widely varies based on their clinical condition, fluid state, diurnal variations, and external environment (Menke and Warnecke, 2004). The present study showed that RNA recovery was high in low-volume samples, but RNA yield and urine volume are not correlated. The clinical disease progression also influenced the volume of the patient with renal disease. Therefore, it is not easy to interpret.

This protocol yielded enough RNA from urine sediment irrespective of the volume ($p > 0.05$) using the centrifugation method, concentrating urine samples before RNA extraction gives a solution to avoid discrepancy. There is a possibility of losing low molecular weight RNA during centrifugation. Nevertheless, standard centrifugation forces with time are needed to optimize the protocol. Additionally, a study was conducted to correlate the RNA yield with a day time variation of sample collection and revealed no significant difference in yield at a different time of collection (Martínez- Fernández *et al*., 2016).

The presence of bacteria in urine is another factor in studying the yield of RNA from urine, which rapidly degrades the RNA. In contrast, in the current study, total RNA recovery was relatively high for samples with positive bacterial cultures than for negative cultures; however, no statistically significant variation was observed between the two groups ($p > 0.05$). The fact was further supported by a study that compared the detectable RNA from healthy and urinary tract infection patients and reported that the detectable RNA was high enough in patients with urinary infections (Menke and Warnecke, 2004).

The modified phenol-based RNA isolation method requires 2 to 3 hours to isolate RNA, especially for low-yield urine samples. The primary benefit of using this method is lower cost than other rapid RNA isolation methods. This method does not require additional purification steps or any chemical preservatives and could be directly used for cDNA synthesis after extraction. In addition, in-house reagent preparations are possible for this protocol, yet additional precautions are needed to avoid RNase contamination and maintain personal safety while working with hazardous chemicals. Unlike rapid kit methods, performing a large batch of samples with minimum reagent cost could be achieved because commercial kits available as 50 preparation or 100 preparations cost relatively high and are limited to sample numbers.

Preservation of samples is necessary to avoid RNA degradation during the pre-analytical phase. It is recommended to store the samples immediately at -80 °C until processing. This can be achieved by snap freezing using liquid nitrogen directly after collection and preventing RNA degradation during transportation. Some commercially available chemical preservatives, such as RNA later, Norgen preservatives or EDTA-containing buffers, prevent RNA degradation at the pre-analytical phase. Using RNA later for urine after concentration could improve the yield of RNA (Medeiros *et al*., 2003). However, it was noted that the present study yielded a comparably equal amount of RNA without using RNA later. The effect of these chemicals on the yield and downstream applications must be studied further before being used. Nevertheless, freezing at -80 °C is easy and more cost-effective than commercial preservatives. However, amorphous phosphate and urate precipitation are unavoidable and interfere with RNA extraction during the freeze-thaw cycle, especially in pathological samples. Certain RNA degradation levels were also recorded in extracted RNA from urine samples. After agarose gel electrophoresis, a single intact band was obtained (**Figure 2**).

Supporting the present study, previous studies by Monteiro *et al.* in 2016 and Tavares *et al*. in 2011 demonstrated a single peak in electropherograms and a single intact RNA band with a certain level of RNA degradation. It is mainly due to the hostile environment of urine itself. However, the present study revealed an acceptable range of Ct values for the *B2M* gene possibility for gene expression analysis. Another drawback of this protocol is that it uses chemical reagents, potentially hazardous and cause mutations. Therefore, special precautions need to be followed when working with these chemicals.

Before validating the protocol, it is recommended to maintain an RNase-free environment from the step of preparing sample collection containers until cDNA synthesis. Commercially available RNase and DNase-free plastic wares could be used throughout the procedures. All the glassware was baked at 300 °C for 4 h before RNA work. Reagents were prepared using 0.05% DEPC treated and autoclaved water (121 °C for 35 min) to maintain a nuclease-free environment. Alternatively, plastic ware could be reused once treated with 0.1 M NaOH/1mM EDTA at 37 °C for 2 h and followed with DEPC treatment, and autoclaving is also cost-effective (Nielsen, 2011). Additionally, to all the above precautions, technical expertise is required to maximize the quantity and quality

of RNA to study transcriptomic analysis irrespective of the disease conditions.

CONCLUSION

In conclusion, a protocol to extract RNA from urine specimens using a modified phenol-chloroform technique was developed and yielded acceptable RNA concentration with high purity for downstream application. The major advantages of these protocols are their cost-effectiveness and high RNA yield compared to other commercial RNA isolation kits. In addition, this protocol does not require pre-and post RNA extraction processes. It, therefore, could be used especially for gene expression studies for large batches of samples with minimum cost. Further, the validation of this protocol using *B2M* as a reference gene resulted in acceptable threshold cycle values from both the control and patient study groups, suggesting suitability for potential biomarker discovery studies for renal disorders like CKD.

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CONFLICTS OF INTERESTS

The authors declare no competing interests among authors.

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