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ORIGINAL ARTICLE

Norepinephrine Effects on the Gene Expression of *LuxS* and *HlyA* of *E.Coli* Isolated from UTI

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ABSTRACT
Urinary tract infections (UTIs) are common and costly bacterial diseases that affect millions of people worldwide. The main cause of UTIs is Escherichia coli (E. coli), a type of bacteria that normally lives in the gut but can also infect other parts of the body. Some E. coli strains have special genes that make them more virulent and resistant to antibiotics. One factor that may influence the severity and outcome of UTIs is stress, which triggers the release of norepinephrine, a chemical messenger that regulates various functions in the body. In this study, we investigated the effect of norepinephrine on E. coli, the main cause of UTIs, in Iraq, a country where UTIs are prevalent and poorly managed. We collected and identified bacterial samples from patients with UTIs and tested them for three genes: 16s RNA, Lux S, and hlyA. The 16s RNA gene is a universal marker for bacterial identification, the Lux S gene is involved in quorum sensing and biofilm formation, and the hlyA gene encodes for alpha-hemolysin, a toxin that damages host cells. We selected one E. coli isolate that had all three genes and measured its gene expression with or without norepinephrine by qRT-PCR. We found that norepinephrine increased the expression of the hlyA gene, which encodes for a toxin. This suggests that norepinephrine enhances the virulence of E. coli and may worsen UTIs in stressed individuals.
Keywords*: Escherichia coli, urinary tract infections, norepinephrine, gene expression, virulence.*

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INTRODUCTION

A urinary tract infection (UTI) is characterized by the presence of bacteria in regions of the urinary tract that are typically devoid of microorganisms, including the kidneys, ureters, bladder, and proximal urethra [26, 6]. UTIs are prevalent, with an estimated annual incidence exceeding 150 million individuals. These infections are regarded as highly detrimental, occupying the second position among bacterial infections [22]. The occurrence of urinary tract infections is contingent upon the virulence of the bacteria responsible and the susceptibility of the host [13]. *Escherichia coli (E. coli*), a Gram-negative bacterium, is commonly found in the human and animal gastrointestinal tract, where it thrives as a facultative anaerobe [10]. The majority of *E. coli* strains is considered part of the normal flora and infrequently lead to clinically significant diseases. Nevertheless, certain strains of *E. coli* possess virulence genes that enable them to induce infections in both the intestinal and extra-intestinal regions of humans and animals [5, 11]. Also *E. coli* is one of the common causes of bacterial infections in patients with chronic kidney disease (CKD), which is associated with oxidative stress [1, 3]. E.coli is the most common bacterial pathogen causing UTI in Al-Najaf city, and it may carry various resistance genes [9].

Catecholamines, namely norepinephrine, epinephrine, and dopamine, are nitrogenous organic compounds that originate from the amino acid L-tyrosine [21]. These compounds are primarily secreted from sympathetic nerve terminals and exhibit a remarkably preserved molecular structure across various vertebrates, encompassing fish, amphibians, and mammals [8]. Stress is a multifaceted phenomenon that disrupts the homeostatic balance of the entire organism. During periods of heightened stress, the levels of stress hormones and the secretion of catecholamines can increase significantly, reaching values that are 20 to 40 times greater than the typical physiological levels [4]. There exists a hypothesis suggesting that the hormones generated as a result of stress exert a significant influence on the infectious capacity of

bacteria. Previous studies have demonstrated that catecholamines possess the ability to facilitate the growth and multiplication of bacteria, along with the promotion of virulence-related characteristics, adhesion properties, and the formation of biofilm. Catecholamines have been found to exert an influence on the outcome of infections caused by these bacteria across different hosts, as demonstrated by Sarkodie et al. [19]. Norepinephrine (NE) is a sympathomimetic amine that is produced from tyrosine and is synthesized in both the adrenal medulla and adrenergic neurons that are found throughout the body [24]. The activation of virulence-associated factors and the enhancement of cytotoxic activity have been observed in various bacterial species, such as *E.coli*, *S. typhimurium*, and *Aeromonashydrophila*, upon exposure to NE [2, 7, 16, 17]. This study was conducted to address the research gap regarding the impact of norepinephrine on UTI-causing *E. coli* in Iraq, as there is a dearth of existing literature on this topic.

MATERIAL AND METHODS

Study Design,Population, and Criteria

A research investigation was conducted at AL-Najaf AL-Ashraf teaching hospital, located in Najaf, Iraq, spanning from September 2022 to May 2023.The study included a diverse group of participants spanning different age groups, ranging from individuals over 18 years old to those under 60 years old. These participants were diagnosed with UTI through clinical evaluation conducted by a specialized physician. All participants in the study had abstained from antimicrobial drug treatment for UTIs in the two weeks prior to the study and had given informed consent to take part. The study excluded female patients who were experiencing menstruation, patients who had recently consumed antimicrobial drugs within a two-week period, patients with chronic illnesses, and patients who had not given their consent.

Sample Collection

A total of 150 urine samples were gathered. Approximately 10 milliliters of morning mid-stream urine samples were aseptically transferred into sterile urine collection tubes. The specimens were subsequently streaked onto MacConkey agar plates utilizing the streaking technique. The plates were subjected to incubation at a temperature of 37 degrees Celsius for a period of 24 hours.

Growth Culturing and E. Coli Response Under Norepinephrine (NE) Treatment

Three separate replicates of the study were performed on three separate days. For each replicate, a loopful of bacteria preserved in glycerol stock was streaked onto MacConkey agar and incubated at 37°C for 18 hours. A single colony was picked up and inoculated into a flask containing 250 ml of sterilized nutrient broth overnight at 37°C with shaking at 250 rpm. From this culture, 500 µL (~ 2.0 x 107 CFU/mL) were used to inoculate 50 ml of nutrient broth and incubated for 4 hours until the growth of bacteria reached a turbidity of~ 0.03 (at 600 nm). At this point, volumes containing 50 µM and 100 µM of NE for treatment samples, or the same volume of vehicle for the control sample, were added and incubated at 37°C for 12 hours. After incubation, cells were harvested directly into a bacteria-protected RNA solution (RNAlate) to preserve RNA from the action of lysate enzymes and kept at 2°C until processed for RNA extraction and subsequent analyses**.**

Total RNA extraction,cDNA synthesis and Rt-qPCR

The total RNA of the samples was extracted using a modified procedure consisting of the boiling method of RNA preparation according to [12] in addition to total RNA extraction kit from (Solar bio)**.**The purity and the concentration (ng/L) of isolated RNA were evaluated by an ultraviolet-visible light spectrophotometer instrument; RNAse-free H_2O was used as a blank measurement, and the absorption at wavelengths 200-700 nm has been saved. The Universal RT-PCR (M-MLV) kit is a complete system for the efficient synthesis of first strand miRNA from total RNA templates. The preparation of cDNA was done according to the manufacturer's instructions. Rt-qPCR technique was performed for these genes: *16s RNA, Lux S,Hemolysin A,*genes in *Escherichia coli* based on specific primers, table (1).

Table 1: Primers information

Primer Name		Sequence (5'-3')	Product Size (bp)	Reference
16s-rna	F	CTTAACAAACCGCCTGCGTG	103	This study
	R	AAGAAGCACCGGCTAACTCC		
LuxS	F	GTCTTCCATTGCCGCTTTCC	169	This study
	R	TACCCTGGAGCACCTGTTTG		
hlyA	F	ATAGTCACTCCCCGTTCGGT	126	This study
	R	TGTCAGGACGGCAGATGAAC		
	R	AGACGTGGACCTCACTCTGA		

SYBR Green PCR Master Mix (Solar bio) were used. The qPCR reactions were carried out in the (QIAGEN) the real-time qPCR conditions were as follows: The DNA template was initially denatured at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 25 seconds. A melting curve analysis was performed from 50°C to 100°C with a 3-minute hold at each temperature to determine the specificity of the amplification.

Statistical Analyses

Data were expressed as means ± standard deviation, statistical analyses were performed using one-way ANOVA followed by Tukey's- test for multiple comparisons by using SPSS version 22 computer program. The $P \leq 0.05$ was considered a significant for all data of the results.

RESULTS

In the present study 150 specimens were collected from patients with urinary tract infections (UTIs) at Al-Najaf Al-Ashraf Teaching Hospital in Al-Najaf province, Iraq, from September 2022 to November 2022. Out of these specimens, 110 (74.33%) showed significant bacterial growth, including 81 (73.64%) from females and 29 (26.36%) from males (Table 4). We identified multiple bacterial species as the causative agents of UTIs, with *E. coli* being the most prevalent (35 isolates).Genomic DNA was extracted from the 35 *E. coli* isolates and tested for the presence of three genes: *16s RNA, Lux S*, and *hlyA*. The *16s RNA* gene is a universal marker for bacterial identification, the *Lux S* gene is involved in quorum sensing and biofilm formation, and the hlyA gene encodes for alpha-hemolysin, a toxin that damages host cells. All isolates (100%) were positive for the *16s RNA* and *Lux S* genes, indicating that they were *E. coli* strains with quorum sensing ability. However, only seven isolates (20%) were positive for the *hlyA* gene, suggesting that they were more virulent than the others.

To investigate the effect of norepinephrine (NE) on the gene expression of *E. coli*, we selected one isolate (no. 6) that tested positive for all three genes by PCR and grew it in nutrient broth media with or without NE at two concentrations: 50 µM and 100 µM. We measured the gene expression of *E.coli* with NE or without NE after 12 hours by qRT-PCR and normalized it to the reference gene 16s RNA. The 12-hour time point was chosen to capture the long-term effects of NE on the gene expression of *E. coli*, which might not be evident at earlier time points.As shown in Figures 1, and 2, NE addition resulted in significant changes in the gene expression of E. coli. The *hlyA* gene was upregulated by 1.62-fold in the presence of NE at 50 µM ($p \leq 0.05$), indicating that NE increased the production of alpha-hemolysin by *E. coli*. However, there was no significant difference in the *hlyA* gene expression between NE at 100 µM and no NE (1.12-fold). The expression of luxS gene was not significantly affected by NE (50µM and 100 µM), as shown by the fold changes of 1.11 and 1.05, respectively ($p > 0.05$).

DISCUSSION

In this study, we identified multiple bacterial species as the causative agents of urinary tract infections, with *E. coli* being the most prevalent (35 isolates). In fact, the *hlyA* gene is present in some strains that can cause UTI, and encodes a hemolysin toxin that can lyse red blood cells and other host cells [15]. The expression of the *hlyA* gene in *E. coli* is regulated by several factors, such as iron availability, temperature, and growth phase [1]. NE may affect the expression of the *hlyA* gene in *E. coli* by modulating iron uptake [23]. Therefore, it is possible that NE addition resulted in significantly upregulated expression of the hlyA gene in 50µM, but not in 100 µM, due to different responses of this bacteria to NE at different doses, and this is a hypothesis that needs further experimental validation, or NE at 100 µM may be toxic to *E. coli* or may activate negative feedback mechanisms that prevent gene induction or inhibit the uptake of iron, which is essential for bacterial growth. The present result confirmed the findings of Sharma *et al.* [20], who reported that norepinephrine induced the expression of many genes that are usually turned on in the stationary phase, plus genes involved in bacterial virulence, stress response, and various metabolic pathways in *E. coli* O157:H7. Moreover, the present study corroborated the observation that norepinephrine increased the expression of bacterio-ferritin and iron-containing proteins was enhanced at transcript level; conversely, the genes involved in amonabactin synthesis and transport were repressed at both transcript and protein levels. in *A. hydrophila* after exposure to NE [17].The present study found that there was no statistical significance ($p > 0.05$) of the expression of *luxS* (1.11-fold) and (1.05-fold) in NE (50µM, and 100 µM) respectively. The *luxS* gene in *E. coli* encodes a synthase for auto inducer 3 (AI-3), a quorum-sensing molecule that can regulate the expression of various genes, including some virulence genes [25]. NE can also affect the expression of some virulence genes in *E. coli* by binding to AI-3 receptors or modulating the production of AI-3 [25]. But in the present result, the effect of NE on the gene expression of this gene wasn't significant; it is possible that norepinephrine does not affect the expression of the *luxS* gene or its downstream targets in some cases. For example, norepinephrine may not have an

effect on *luxS* gene expression if the AI-3 receptors are saturated or inhibited by other molecules, or if the luxS gene is already maximally expressed or repressed by another factor [24]. Alternatively, norepinephrine may not affect the expression of some virulence genes that are regulated by the *luxS* gene if those genes are also controlled by other regulators that override the effect of the luxS gene. At last the current study suggests that norepinephrine, a stress hormone that is elevated in patients with trauma, shock, or intensive care, may increase the expression of *HlyA* gene in *E. coli*, making them more virulent and resistant to antibiotics.

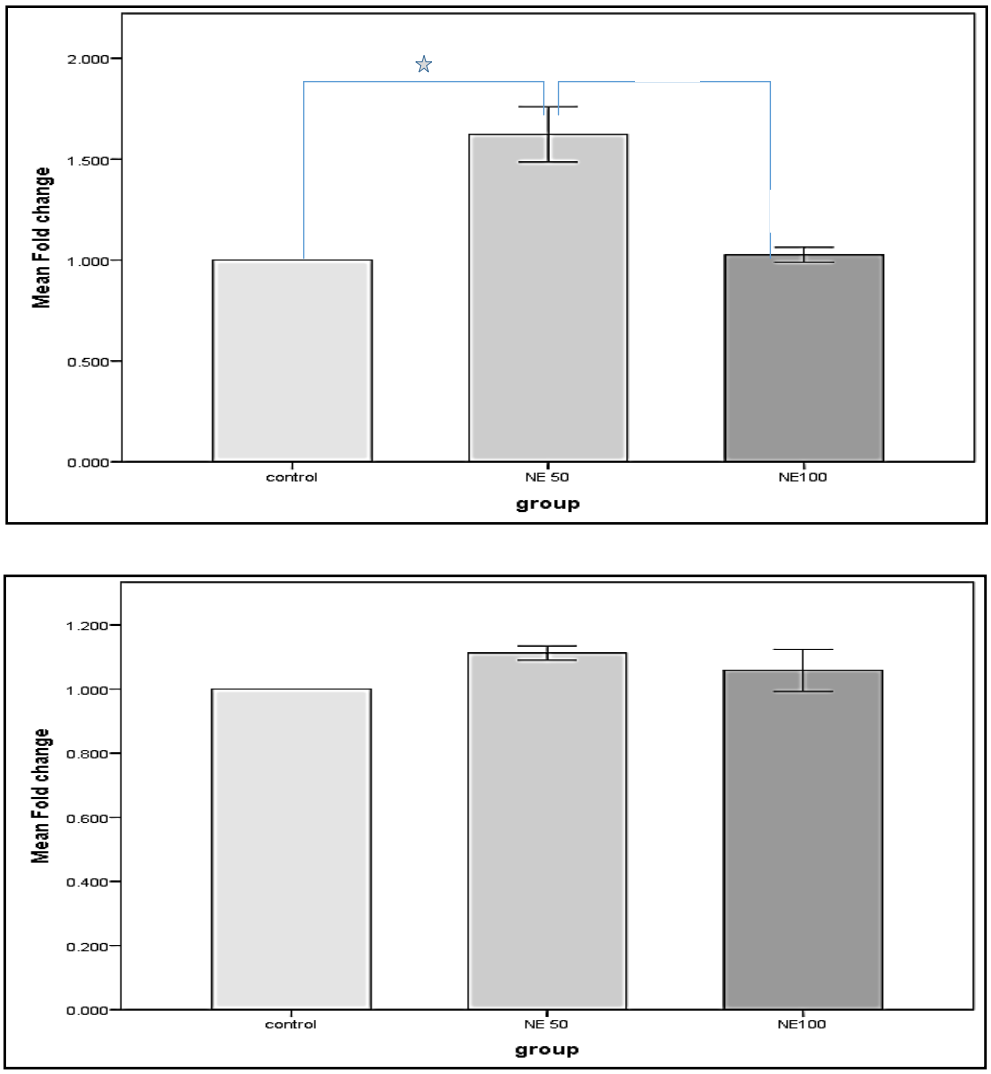

Figure 1 Bar graph presentation of the fold change of virulence gene expression analyzed by RT-qPCR, normalized to the 16S rRNA (housekeeping gene), and expressed as a change when compared to their respective controls. (A)hlyA gene; (B) LuxS gene. The data presented are mean± standard deviation (Error bars). * indicates a significant difference from the control group; (#) indicates a significant difference from the second group. Tukey's- test; NS: not -significant; $p \leq 0.05$

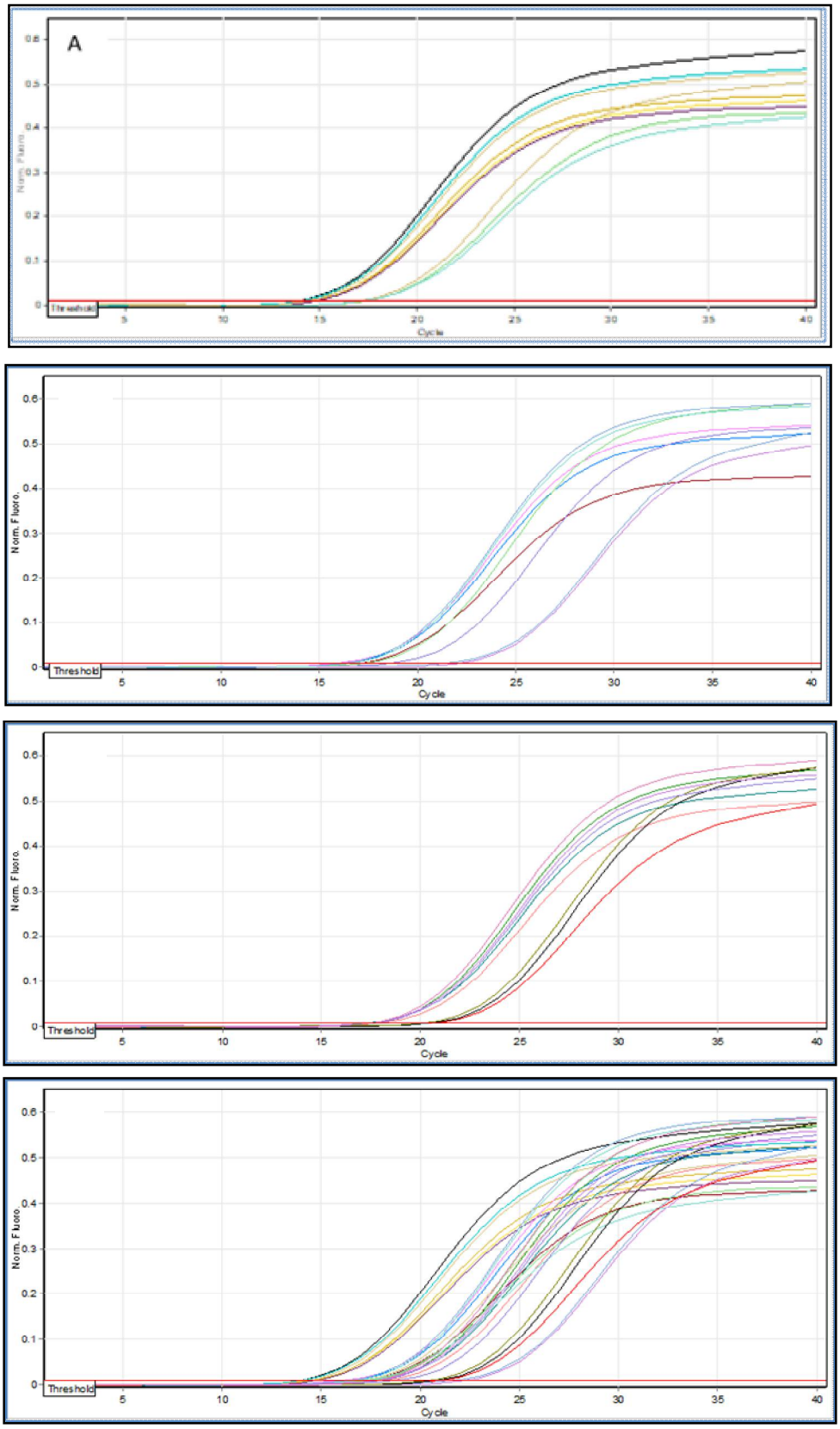

Figure 2 RT-qPCR amplification plots after treatment with or without norepinephrine (50µM, and 100 µM) (A) 16SrRNA gene (B) Hly A gene (C) Lux S gene (D) the raw data for amplification plots of all sequential samples. SYBR Green dye, FAM channel.

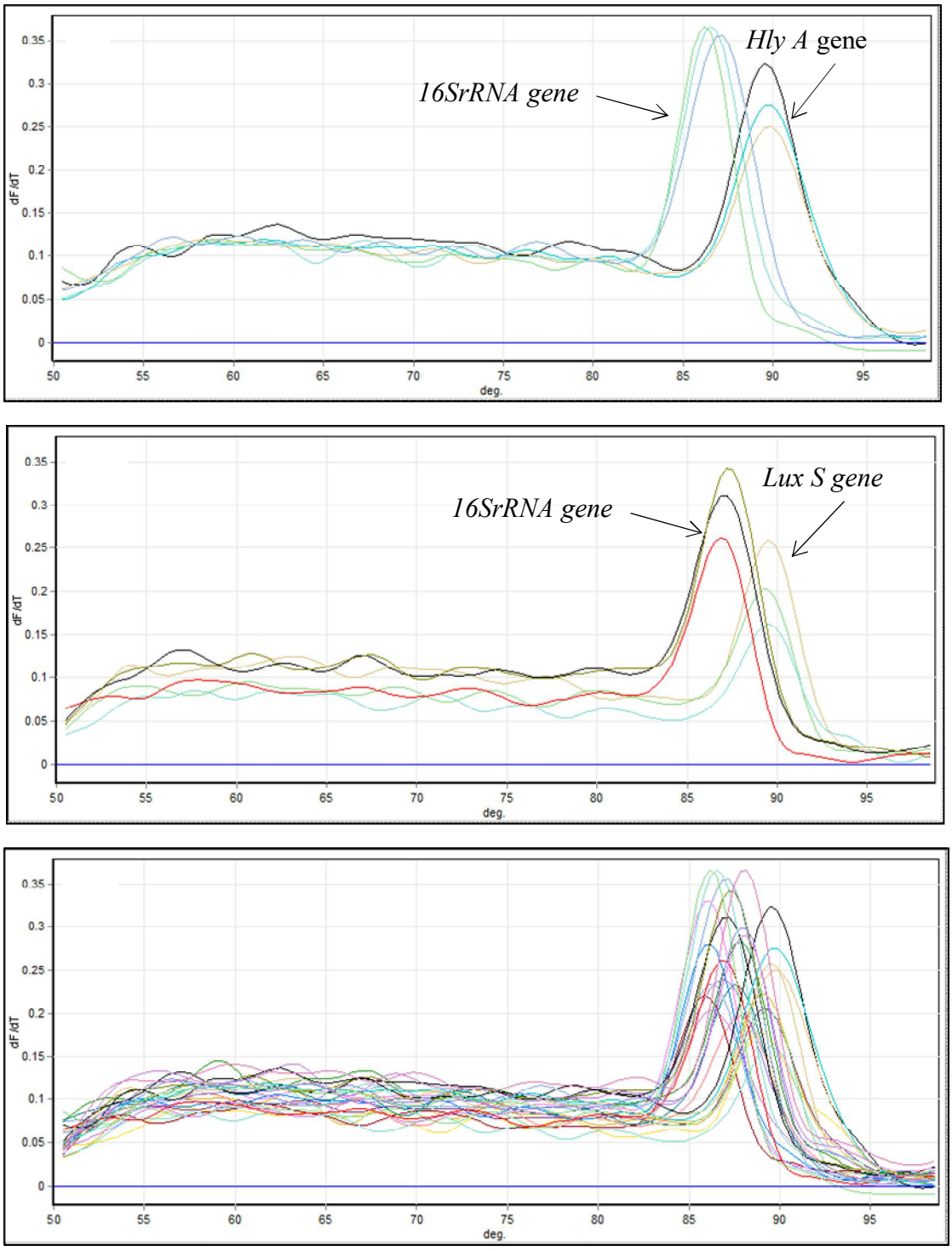

Figure 3: Melting curve analysis of RT-qPCR amplified genes normalized to 16S rRNA gene showing (A) hlyA gene, (B) LuxS gene, and (C) raw data for plots of all sequential samples; these curves indicated no primer dimers; primer dimers can interfere with the accuracy and specificity of qRT-PCR results. SYBR Green, (FAM)channel.

CONCLUSION

In conclusion, we demonstrated that norepinephrine affects the gene expression and virulence of *E. coli*, the main cause of UTIs, in Iraq. Our findings suggest that stress may worsen UTIs by enhancing the

production of toxins by *E. coli.* However, **the** study had some limitations, such as the small sample size, the use of only one *E. coli* strain, and the lack of clinical data. Future studies should use more samples, strains, and methods to confirm and extend our results.

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