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Evaluation of *Escherichia coli* aggR Gene Expression in the Presence of Different Concentrations of Creatinine

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Abstract

Background: Chronic renal failure is an irreversible disease associated with elevated blood creatinine levels and substantial mortality and morbidity in children worldwide. Enteroaggregative *Escherichia* coli (EAEC) is a diarrheagenic agent that possesses several virulence factors, including aggR.

Objectives: This study aimed to evaluate aggR gene expression in EAEC under different levels of creatinine exposure.

Methods: Initially, three biochemically confirmed *E. coli* isolates obtained from medical laboratories in Borujerd city, western Iran, were molecularly evaluated by 16S ribosomal RNA (16S rRNA) gene amplification. Next, the isolates were assessed for the aggR gene using polymerase chain reaction (PCR). Isolates carrying the aggR gene were cultured in Brain Heart Infusion (BHI) medium supplemented with 1, 3, and 6 mg/dL creatinine (37^{O}C for 24 hours). The expression level of aggR was evaluated in creatinine-exposed isolates using real-time PCR.

Results: Among 50 clinical *E. coli* isolates, three were EAEC, and one of these isolates carried the aggR gene. Real-time PCR showed that aggR gene expression decreased significantly in the presence of varying creatinine concentrations ($P < 0.0001$).

Conclusions: These findings warrant further investigation to elucidate the role of creatinine in modulating bacterial virulence gene expression in the context of renal disorders.

Keywords: Enteroaggregative *Escherichia coli*, aggR, Creatinine, Real-time PCR

1. Background

Diarrheal illnesses caused by diverse infectious agents constitute a major public health concern in both adults and children (1, 2). *Escherichia coli*, a gram-negative, rod-shaped bacterium capable of facultative anaerobiosis, is a prominent diarrhea-causing pathogen among the infectious agents commonly detected in the human intestinal tract (3). Although *E. coli* is typically part of the normal gut microbiota, certain strains can acquire virulence factors and become pathogenic variants, leading to serious diseases such as diarrhea. These pathogenic strains are classified into 6 distinct pathotypes according to their virulence mechanisms (4). Enteroaggregative *E. coli* is a major cause of both acute and persistent diarrhea across all age groups, particularly in infants and immunocompromised individuals (5-7). The clinical presentation of Enteroaggregative *Escherichia* coli (EAEC) infection often includes fever, anorexia, nausea, and bloody-mucosal diarrhea (8). The classic diagnostic method for EAEC involves identifying the characteristic "stacked-bricks" aggregation pattern in HEp-2 cell culture (9). The pAA virulence plasmid is a key molecular marker of EAEC and contains essential pathogenicity genes, including aggR, aatA, and genes encoding aggregative adherence fimbriae (10, 11).

The aggR gene functions as a master regulator of EAEC virulence. Strains carrying this gene are classified as typical EAEC and are generally more virulent, causing more severe disease than atypical strains (12, 13). EAEC pathogenesis is a complex process involving initial adherence to host cells, biofilm formation, and subsequent toxin release that triggers an inflammatory response (14). In addition to diarrheal disease,

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diarrheagenic *E. coli* strains, including EAEC, have recently been linked to urinary tract infections (UTIs), which affect more than 150 million people worldwide (15-18). The urinary tract provides an environment in which uropathogenic microorganisms can thrive, and their growth can be influenced by the chemical composition of urine, including the concentrations of urea and creatinine. In patients with renal insufficiency, host-pathogen interactions are markedly altered by the accumulation of various substances, which in turn affects microbial virulence factor expression and host defense mechanisms. Notably, studies have shown that high creatinine levels can inhibit bacterial persistence in the urinary tract (19).

2. Objectives

This study aimed to examine the effect of varying creatinine levels on aggR gene expression in clinically isolated *E. coli* strains from individuals with urinary tract infections in Boroujerd, western Iran.

3. Methods

3.1. Collection and Maintenance of Clinical Isolates

In total, 50 *E. coli* strains were obtained from clinical samples from individuals with urinary tract infections at diagnostic medical facilities in Borujerd, Lorestan province. The identity of these isolates had been previously confirmed using biochemical methods. All isolates were preserved at -20°C in Brain Heart Infusion broth supplemented with 15% glycerol for future analysis (20).

3.2. DNA Extraction and Amplification

Genomic DNA was extracted using the boiling method. Briefly, bacterial cultures were grown from glycerol stocks in BHI broth and subcultured on blood agar. Individual colonies were then transferred to BHI broth and incubated for 20 hours. After incubation, cells were pelleted, resuspended in 500 µL of distilled water, and boiled at 95°C for 5 minutes to induce lysis. After centrifugation, the supernatant containing genomic DNA was collected. DNA concentration was measured at 280 nm using a spectrophotometer, and samples were subsequently stored at -20°C (21).

3.3. PCR-Based Identification and Virulence Gene Detection in Clinical Bacterial Isolates

3.3.1. Species Identification via 16S rRNA Gene Amplification

Clinical isolates were identified by targeting the 16S rRNA gene. PCR amplification was performed in a total volume of 25 µL, including 12.5 µL of Ampliqon MasterMix (Ampliqon, Denmark), 1 µL each of forward and reverse primers (5 pmol), 4 µL of purified DNA, and 6.5 µL of sterile distilled water. Amplification was performed using a thermocycler under the following conditions: initial denaturation at 95°C for 1 minute, followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 20 seconds, with a final extension at 72°C for 7 minutes (22).

3.3.2. Virulence Gene Screening for aggR

To detect the aggR gene, standard PCR was used. The amplification mixture was prepared as described earlier. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and elongation at 72°C for 40 seconds, concluding with a final elongation at 72°C for 7 minutes. The resulting amplicons were resolved by electrophoresis on a 1.5% agarose gel using a 50-bp molecular weight marker as a reference. Bands were visualized by UV transillumination. Details of the oligonucleotide primers used to amplify the 16S rRNA and aggR genes are provided in Table 1. Positive amplification results confirmed bacterial strain identity and determined the presence of the aggR virulence factor in the isolates (23, 24).

3.4. in vitro Assessment of Creatinine Effects on aggR-Positive E. Coli

To assess the effects of creatinine on aggR-positive *E. coli* clinical isolates, as confirmed by prior PCR screening, an in vitro assay was performed. The selected creatinine concentrations (1, 3, and 6 mg/dL) were chosen to simulate serum levels commonly observed across the spectrum of renal function: approximately the upper limit of normal in healthy individuals (approximately 1 mg/dL), moderate chronic kidney disease (approximately 3 mg/dL), and severe/advanced chronic kidney disease or pre-dialysis stages (approximately 6 mg/dL) (25, 26). Creatinine was dissolved in sterile distilled water at concentrations of 1, 3, and 6 mg/dL to prepare BHI broth, with the appropriate amount of BHI powder added according to the manufacturer's instructions for the volume of water used. The mixtures were sterilized using 0.22-µm syringe filters (Merck Millipore, Darmstadt, Germany) and inoculated with the isolates. Cultures were incubated at

Table 1. Primer Pairs Used for Amplification of the 16S rRNA and aggR Genes in EAEC Clinical Isolates Involved in Urinary Infections in Medical Laboratories in Boroujerd, Western Iran

Genes	F/R	Primer Sequence (5' -> 3')	Product Length	Reference
aggR	F	GAATCGTCAGCATCAGCTACA	102 bp	(39)
	R	CCTAAAGGATGCCCTGATGA		
16S rRNA	F	AGTTATCCCCCTCCATCAGG	99 bp	(40)
	R	TGCAAGTCGAACGGTAACAG		

37°C for 24 hours under aerobic conditions to evaluate the response of the isolates to creatinine.

3.4.1. RNA Extraction and Real-Time PCR

RNA was extracted from 24-hour cultures of aggR-positive *E. coli* clinical isolates before and after creatinine exposure using a commercial RNA extraction kit (CinnaGen, Iran), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 25 µL of purified RNA using the CinnaClone kit (CinnaGen, Iran). The cDNA was amplified by conventional PCR in a 25-µL reaction mixture containing 12.5 µL of MasterMix (Ampliqon, Denmark), 3 µL of cDNA, 1 µL each of forward and reverse primers for a housekeeping gene (5 pmol), and 7.5 µL of sterile distilled water, with thermocycling conditions of initial denaturation at 95°C for 1 minute, 45 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 25 seconds, and extension at 72°C for 20 seconds, followed by a final extension at 72°C for 1 minute. Amplified cDNA was stored at -20°C for subsequent use. Real-time PCR was performed to quantify aggR gene expression using an ABI StepOne system (Applied Biosystems, USA) in a 15-µL reaction volume comprising 7.5 µL of Real-Time MasterMix, 5.1 µL of DEPC-treated water, 0.7 µL each of specific forward and reverse primers, and 1 µL of cDNA. Amplification included a holding stage at 94°C for 5 minutes, 45 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 40 seconds, followed by a melt-curve stage with steps at 95°C for 15 seconds, 58°C for 30 seconds, and 95°C for 15 seconds. Reactions were performed in duplicate, with a housekeeping gene as a positive control, and data were analyzed using REST software.

4. Results

4.1. PCR Amplification

Purified DNA samples from *E. coli* clinical isolates showed distinct bands on 1% agarose gel electrophoresis, confirming successful extraction (Figure 1A). PCR amplification of the 16S rRNA gene (99 bp) confirmed the molecular identity of the *E. coli* isolates (Figure 1B). Among the three EAEC isolates, only one tested positive for the aggR gene (102 bp) by PCR (Figure 1C). RNA samples exhibited an A260/A280 absorption ratio of 1.8 - 1.9, indicating high purity, and gel electrophoresis showed distinct 16S and 23S rRNA bands, confirming RNA integrity and purity (Figure 2A).

4.2. cDNA Validation and aggR Gene Expression Analysis in Creatinine-Treated E. Coli

Synthesized cDNA from EAEC clinical isolates obtained from patient samples in Borujerd city was validated by agarose gel electrophoresis, which showed clear, distinct bands, confirming successful cDNA synthesis and integrity (Figure 2B). Quantitative reverse transcription PCR results revealed marked and statistically significant downregulation of the aggR gene in bacterial strains after treatment with creatinine at concentrations of 1, 3, and 6 mg/dL ($P < 0.0001$), indicating a clear dose-responsive pattern (Figure 3A and B). The expression levels of aggR messenger RNA (mRNA) were relatively quantified, showing consistent downregulation across the tested creatinine concentrations compared with untreated controls, with the most pronounced reduction observed at the highest concentration of 6 mg/dL (Figure 3B). These findings are summarized in Table 2, which details the fold changes in aggR mRNA expression for each isolate and concentration, highlighting variability among the isolates and the significant impact of creatinine treatment on virulence gene expression.

5. Discussion

E. coli, commonly regarded as a harmless resident of the human gut microbiome, can transform into disease-causing forms, including EAEC, a prominent contributor to global diarrheal illness (27, 28). Since the initial identification of bacteria, researchers have sought effective therapies to combat the infections they cause. However, the emergence of antibiotic resistance has created substantial obstacles to treating illnesses caused

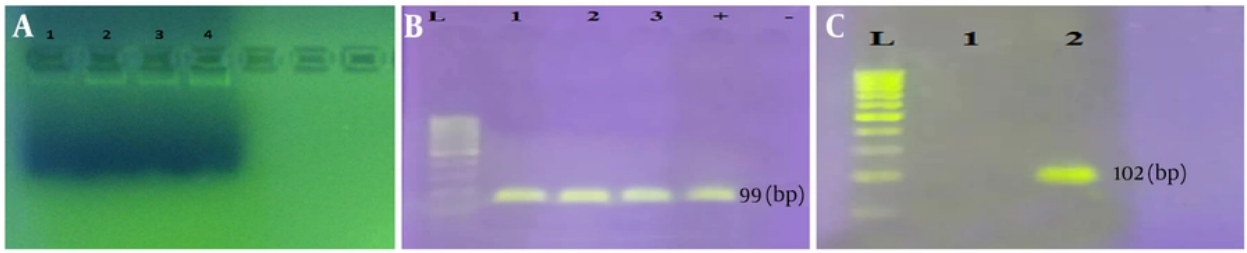

Figure 1. A, Validation of the extracted DNAs from EAEC clinical isolates involved in urinary infections in medical laboratories in Boroujerd, west of Iran. B, Identification of the 16S rRNA gene fragment (length: 99 bp) in EAEC clinical isolates. C, Amplification of the aggR gene (length: 102 bp) in the EAEC clinical isolates. DNA marker: 50 bp.

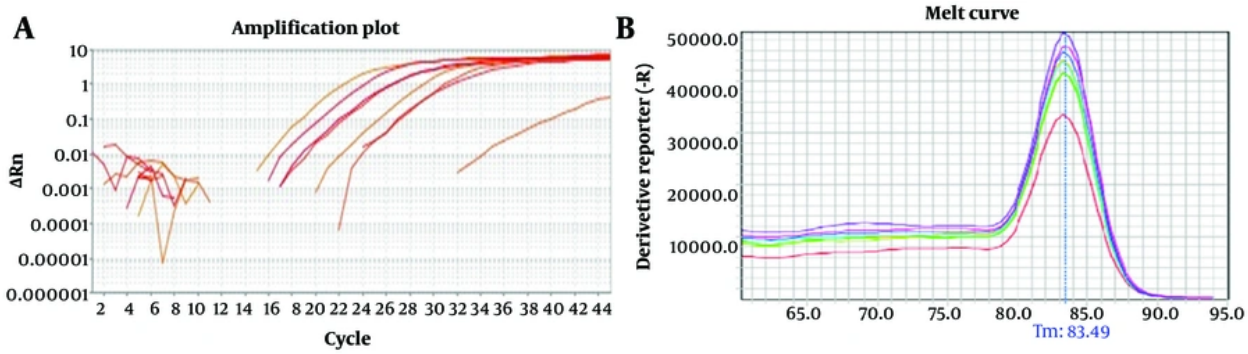

Figure 2. A, Quality of the purified RNAs in negative control and samples treated with creatinine concentrations (1, 3, and 6 mg/dL) (lanes 1 - 4); two specific 16S and 23S RNA bands are indicative of the integrity and purity of the extracted RNA. B, cDNA quality control.

Table 2. Relative Quantification Analysis Showing the Expression Level of aggR Gene mRNA in EAEC Clinical Isolates Involved in Urinary Infections in Medical Laboratories in Boroujerd, Western Iran

Genes	Reaction Efficiency	Expression	Result	P-Value
aggR1	0.976	0.000	Decrease	0.0001
aggR2	0.976	0.002	Decrease	0.0001
aggR3	0.976	0.009	Decrease	0.0001
16S	0.9887	1.000	-	-

by diverse microorganisms (29, 30). *E. coli* is a leading cause of numerous human bacterial infections, and multidrug resistance poses a severe threat, particularly among inpatients (31). This organism is a key factor in urinary tract infections and nosocomial conditions such as sepsis, wound complications, gastrointestinal disorders, and infant meningitis. Moreover, *E. coli* acts as an opportunistic pathogen in healthcare settings and has developed resistance to beta-lactam drugs through plasmid-mediated extended-spectrum beta-lactamases (32-34).

The present study observed significant dose-dependent downregulation of aggR gene expression in EAEC clinical isolates from Borujerd city, with reductions evident at creatinine concentrations of 1, 3, and 6 mg/dL ($P < 0.0001$) and most pronounced at 6 mg/dL. These findings highlight creatinine's potential as a modulator of bacterial virulence, potentially through interference with the transcriptional regulation of the

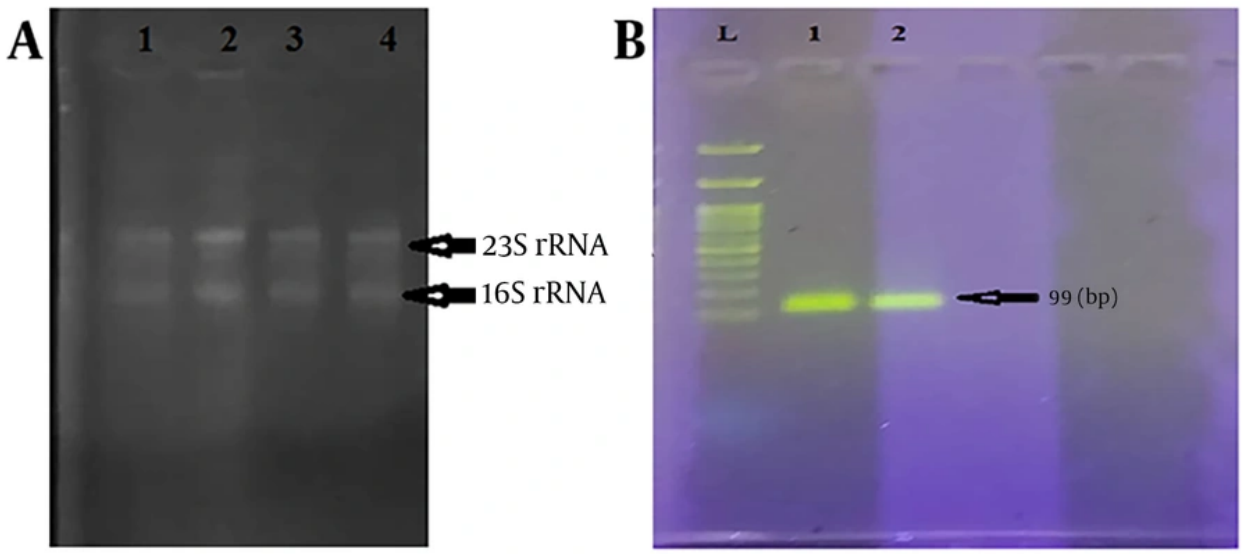

Figure 3. A, Amplification plot of the aggR gene in the isolated EAEC clinical sample involved in urinary infections in medical laboratories in Boroujerd, west of Iran (ΔRn cycle). B, Melt-curve analysis to ensure the specificity of the amplified aggR gene fragment. The amplification peaks are aligned with each other.

aggR regulon on the pAA plasmid. The creatinine concentration range was selected based on reported serum levels in patients with chronic kidney disease to better reflect the physiological/pathological conditions of patients with chronic kidney failure and to enhance the translational potential of the findings to clinical settings. This observation aligns with prior research demonstrating the antibacterial effects of creatinine, such as that of McDonald et al. (19), who reported that creatinine hydrochloride inhibits the replication of diverse bacteria, including *E. coli*, by overwhelming proton efflux mechanisms, suggesting a mechanistic basis for the observed gene expression changes beyond mere growth inhibition.

Similarly, Janbakhsh and Janbakhsh et al. observed alterations in rfbE gene expression in *E. coli* O157:H7 under varying creatinine levels, with initial decreases at low concentrations (1 mg/dL) followed by increases at higher concentrations (3 and 6 mg/dL), indicating that creatinine may exert concentration-specific effects on virulence genes and potentially exacerbate renal pathology in conditions such as hemolytic uremic syndrome through enhanced endotoxin production (35). In contrast, Meiland et al. (36) found no association between *E. coli* bacteriuria and long-term renal function decline in women, implying that asymptomatic bacteriuria alone may not drive kidney damage. However, our results suggest that elevated creatinine in chronic kidney disease could interact with EAEC virulence in comorbid scenarios, particularly in Iran, where chronic kidney disease prevalence is rising dramatically, from 97,300 cases in 1990 to a projected 423,300 by 2030 (37).

The detection of aggR in only one of three isolates (33% prevalence) is consistent with regional variations in EAEC virulence reported globally, such as 66% in Iranian patients reported by Bouzari et al. (38), underscoring the need for broader molecular surveillance in Borujerd. Clinically, these findings suggest that the modulatory effects of creatinine could mitigate EAEC-driven diarrhea in patients with chronic kidney disease and elevated serum levels, such as 3.8 - 7.2 mg/dL in Iranian chronic kidney disease cohorts reported another study; however, the in vitro design and limited sample size limit extrapolation to in vivo contexts.

5.1. Study Limitations and Conclusions

A major limitation of the present study is the small number of isolates evaluated for aggR gene expression (only one aggR-positive isolate), which primarily resulted from the low prevalence of typical EAEC among the collected clinical strains. Therefore, the results should be considered preliminary and exploratory, and further studies using a larger number of aggR-positive isolates are required to confirm these findings and to

investigate potential strain-specific variations in response to creatinine.

This investigation sought to explore the impact of different creatinine levels (1, 3, and 6 mg/dL) on aggR gene expression in clinical *E. coli* strains isolated from Borujerd, Lorestan province, western Iran. The findings showed that three *E. coli* strains were confirmed by PCR targeting the 16S rRNA gene, with one carrying the aggR gene. Notably, this work provides initial evidence that aggR gene expression is suppressed in vitro across varying creatinine doses. These observations highlight the need for additional studies to clarify how creatinine influences the regulation of bacterial virulence genes. Future research should prioritize examining the expression of other virulence elements and genes under creatinine exposure, analyzing target gene activity in stool specimens from individuals undergoing dialysis, and expanding the cohort size to improve statistical reliability. These efforts could provide deeper insights into the interplay among creatinine concentrations, microbial pathogenicity, and health implications for individuals with kidney impairment.

Footnotes

AI Use Disclosure: The authors declare that no generative AI tools were used in the creation of this article.

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